Department of Agricultural Sciences
University of Helsinki
and
Finnish Doctoral Program in Plant Science
Finland

Microtubule and replication vesicle associations of the potyviral HCpro protein

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ACADEMIC DISSERTATION

To be presented, with the permission of the Faculty of Agriculture and Forestry of the University of Helsinki, for public examination in lecture room B3, B-building (Latokartanonkaari 7-9), on 27 September 2013, at 12 noon.

Helsinki 2013

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Cover figure: Interactions of viral HCpro and cellular HIP2 proteins in leaf epidermal cells. Fluorescence signals in green show the localization of interacting HCpro and HIP2. Blue signals indicate chloroplasts and red signals microtubules (upper right corner) or viral replication vesicles (two images in the lower right corner).

ISBN 978-952-10-9037-0 (pbk.)
ISBN 978-952-10-9038-7 (PDF)

Helsinki University Printing House
Helsinki 2013

ABSTRACT

Viruses, as intracellular parasites, occupy and rearrange the host cell. Potyviruses (genus *Potyvirus*), the largest group of plant RNA viruses, infect plants of several families, including the nightshade family (Solanaceae). Potyviruses modify the internal organization of host cell membranes and membranous organelles for the purposes of viral multiplication and movement. At the same time, they have to deceive the receptors of the host cell that guard against external and internal alterations. Virus factories are formed in the perinuclear regions by deploying and concentrating endoplasmic reticulum, Golgi and chloroplasts. The peripheries of the cell, containing cortical microtubules (MTs), are involved in secretion and endocytosis-mediated defence signalling.

Helper component proteinase (HCpro) is a multifunctional protein of potyviruses that participates in subversion of host defence, including RNA silencing. HCpro of *Potato virus A* (PVA) interacts with HCpro-interacting protein 2 (HIP2) of potato (*Solanum tuberosum*) and also with viral proteins that accumulate in or near viral replication vesicles. Host eukaryotic translation initiation factors eIF4E or eIF(iso)4E are required for replication, accumulation or systemic movement of potyviruses and are recruited to viral replication vesicles during infection. In this study, the interaction of HCpro with HIP2 and a new interaction of HCpro with eIF4E proteins were studied and their localizations in infected cells were described. Structural predictions of HCpro and HIP2 were applied for more detailed understanding of these interactions.

HIP2 from potato and tobacco (*Nicotiana tabacum)* were found to be functionally related to a homologous MT-associated protein of *Arabidopsis thaliana*, SPIRAL2. Virus-induced silencing of *HIP2* in *Nicotiana benthamiana* caused twisted growth, a phenotype typical of defects in cortical MT organization. A similar spiral phenotype of an *Arabidopsis spiral2* line was complemented by transgenic expression of potato HIP2. Self-interactions regulated localization and spatial distribution of HIP2 on the cortical MT array. Predicted structural domains, localizations and self-interactions of the plant-specific HIP2 and SPIRAL2 suggested similarities to the eukaryotic multi-TOG-domain proteins CLASP and MAP215 that regulate MT dynamics, and its interactions with the plasma membrane and cell organelles, and participate in endosomal signalling.

Interactions of HCpro and the host proteins were studied in PVA-infected plant cells using bimolecular fluorescence complementation (BiFC). Signals of HIP2 and HCpro interaction occurred in the cell cortex, along MTs and at MT intersections, but also in granules near the chloroplasts. The signals of HCpro interaction with eIF(iso)4E were concentrated in foci that associated with chloroplasts and viral replication vesicles. Replication vesicles of PVA were detected near chloroplasts and occasionally with HCpro and HIP2 at cortical MTs.

Detailed yeast two-hybrid system (YTHS) analysis of HCpro interactions showed that those with HIP2 were regulated by a highly variable region (HVR) in a

hinge region of a central domain of HCpro. Sequence comparisons and YTHS assays revealed a conserved eIF4E binding motif (4EBD) at the beginning of a carboxyl-terminal domain of HCpro. Structural models of HCpro made *in silico* suggested that the HVR formed an exposed loop or coil structure, while 4EBD was within an alpha-helical structure.

Accumulation of PVA was significantly reduced in the *HIP2*-silenced leaves of *N. benthamiana*. Similarly, mutations in HVR of HCpro reduced interaction with HIP2 and accumulation of PVA in potato and *Nicotiana* plants. The silencing-suppression capacity of HVR-mutated HCpro was increased (in 3 types of mutants) or strongly reduced (in one type). The single HVR mutant type that compromised the ability of HCpro to suppress silencing or interact with HIP2 in YTHS greatly reduced accumulation of PVA, but did not prevent replication and movement of the mutated virus. These results confirmed that interaction of HCpro with HIP2 enhances virus accumulation.

Three of the HVR-mutated viruses were hypervirulent, causing necrotic symptoms novel to PVA in systemically infected leaves of *Nicotiana*. A microarray analysis showed activation of the hypersensitive response and signalling by jasmonic acid and ethylene. Structural modelling of HCpro indicated that mutations in HVR altered the conformation of its whole hinge region while the fold of 4EBD remained unaffected. Together with aberrant or disrupted HIP2 interactions, the conformational changes may have triggered host defence.

Several discoveries were made during the course of this thesis. It was found, for instance, that the HCpro interactions with HIP2 are determined by a structurally important but variable region, while those with eIF4E proteins are determined by a highly conserved motif, 4EBD, that is amongst the first of its kind identified in viruses. It was also found that HIP2 has similarities to conserved eukaryotic MT proteins involved in MT dynamics and signalling. MTs are a well-exploited area of research in animal virology, but this is the first report of a role of an MT interaction in virus accumulation in plants and also the first report of an interaction of a potyvirus protein with an MT-associated protein, and its localization with MTs. Considering that HCpro located both at MTs and near the viral replication vesicles, and that it interacted with eIF4E proteins targeted to those vesicles, HCpro may be involved in allocation of intracellular resources or regulation of host signalling for sustained infection. Future developments have potential for the improvement of virus resistance in plants.

Tiivistelmä

Potyvirukset ovat suuri virusryhmä, joka aiheuttaa sato- ja laatutappioita kaikissa viljelykasveissa, mm. perunalla (Solanaceae-heimo). Virustauteja ei voida torjua kemiallisesti, joten virusvapaa lisäysmateriaali ja perinnöllinen viruskestävyys ovat kasvinsuojelun kulmakiviä. Virukset ovat solunsisäisiä loisia, jotka käyttävät ja muokkaavat solun rakenteita ja resursseja lisääntymiseensä ja samalla tyrehdyttävät solun viruspuolustuksen. Täten virusten ja kasvien tuottamien proteiinien väliset suorat vuorovaikutukset ovat avainasemassa kasvien alttiuden ymmärtämiseksi ja kestävyysominaisuuksien löytämiseksi.

Potyvirusten perimän monistus eli replikaatio tapahtuu solulimakalvostosta irtoavissa vesikkeleissä, joita kuljetetaan viherhiukkasten läheisyyteen. Potyvirusten monitoimintainen HCpro-proteiini toimii viruksen monistumisessa ja häiritsee kasvin puolustusta, etenkin RNA-hiljennystä, joka on virusten torjunnan perusmekanismi. Perunan A-viruksen (PVA) HCpro-proteiini kykenee tarrautumaan perunan HIP2-mikrotubulusproteiiniin. Kasvisoluissa mikrotubulukset muodostavat solukalvon alaisen verkoston, joka osallistuu soluelinten ankkuroitumiseen ja solun puolustuksen herättäviin viestiketjuihin. Joitakin kasviproteiineja tiedetään kaapattavan replikaatiovesikkeleihin ja tarvittavan viruksen lisääntymiseen. Tällaisia ovat translaation aloitustekijä eIF4E proteiinit, joiden viruksille sopimattomat muodot tekevät kasvista virusta kestäviä.

Tässä työssä tutkittiin PVA:n HCpro-proteiinin ja kasviproteiinien vuorovaikutusta elävissä, viroottisissa kasvisoluissa. Niiden solunsisäistä sijaintia havainnoitiin fluoresoivien merkkiproteiinien avulla. HCpro-proteiini oli PVA:n tartuttamissa soluissa kosketuksissa sekä mikrotubulusproteiini HIP2:n että translaatiotekijä eIF4E:n kanssa. Vuorovaikuttaessaan eIF4E:n kanssa HCpro sijaitsi viruksen replikaatiovesikkelien läheisyydessä. HIP2:n yhteydessä HCpro sijaitsi mikrotubuluksilla ja mikrotubulusverkostossa havaittiin replikaatiovesikkeleitä.

Vuorovaikutuksille tärkeitä proteiinirakenteita tarkasteltaessa löydettiin HCpro:ssa seitsemän aminohapon pituinen alue, joka säätelee vuorovaikutusta eIF4E:n kanssa ja jonka aminohapot ovat konservoituneita potyvirusten välillä. HIP2-vuorovaikutusta sääteli muunteleva alue. Kun kasvin HIP2-proteiinin määrää vähennettiin estämällä ao. geenin ilmentymistä tai muutettiin viruksen HCpro-proteiinia HIP2:een heikommin tarttuvaksi, HIP2:n voitiin todeta olevan välttämätön PVA:n tehokkaalle monistumiselle. HIP2-vuorovaikutuksen suhteen heikennetty PVA herätti kasvin puolustuksen, johtaen kuolio-oireisiin. HIP2-proteiinin rakenteessa havaittiin yhteneväisyyttä mikrotubuluksia sääteleviin ja solunsisäisen viestinnän proteiineihin. Rakennemallinnuksen perusteella HCpro-muunnokset muuttivat HCpro:n laskostumista. Osa muunnetuista HCpro-proteiineista esti RNA-hiljennystä pidempään kuin muuntamaton HCpro. Nämä HCpro-muodot lisäsivät lehdissä tuotetun vieraproteiinin saantoa, mitä voidaan soveltaa esimerkiksi lääkeaineiden tuotantoon kasveissa.

Saatua uutta tietoa HCpro:n vuorovaikutuksista HIP2:n ja eIF4E:n kanssa voidaan käyttää näiden proteiinien virukselle sopimattomien geenimuotojen etsimiseksi ja sellaisten hyödyntämiseksi viruskestävien kasvien jalostuksessa.

CONTENTS

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications. They are referred to in the text by their roman numerals.

I **Haikonen T**, Rajamäki M-L, Valkonen JPT. 2013. Interaction of the microtubule-associated host protein HIP2 with viral helper component proteinase is important in infection with *Potato virus A*. *Molecular Plant-Microbe Interactions* **26**, 734–744.

II **Haikonen T**, Rajamäki M-L, Tian Y-P, Valkonen JPT. 2013. Mutation of a short variable region in HCpro protein of *Potato virus A* affects interactions with a microtubule-associated protein and induces necrotic responses in tobacco. *Molecular Plant-Microbe Interactions* **26**, 721–733.

III **Haikonen T**, Rajamäki M-L, Valkonen JPT. 2013. Improved silencing suppression and enhanced heterologous protein expression are achieved using an engineered viral helper component proteinase. *Journal of Virological Methods* **193**, 687–692.

IV Ala-Poikela M, Goytia E, **Haikonen T**, Rajamäki M-L, Valkonen JPT. 2011. Helper component proteinase of the genus *Potyvirus* is an interaction partner of translation initiation factors eIF(iso)4E and eIF4E and contains a 4E binding motif. *Journal of Virology* **85**, 6784–6794.

ABBREVIATIONS

4EBD	eIF4E binding domain
6K1	6 kilodalton (kDa) protein 1
6K2	6 kDa protein 2
aa	amino acid
approx.	approximate
ARM	armadillo
At	*Arabidopis thaliana*
BiFC	bimolecular fluorescence complementation
BNYVV	*Beet necrotic yellow vein virus*
C-	carboxyl-
cv.	cultivar
CC	coiled-coil
CLASP	cytoplasmic linker protein 170 associated protein
ClYVV	*Clover yellow vein virus*
CI	cylindrical inclusion protein
CP	coat protein
dpi	days post-inoculation
dsRNA	double-stranded RNA
EB1	end-binding 1
EF3	elongation factor 3
eIF	eukaryotic translation initiation factor
eIF(iso)	isoform of eukaryotic translation initiation factor
ELISA	enzyme-linked immunosorbent assay
ER	endoplasmic reticulum
ERES	ER-exit site
ET	ethylene
ETI	effector-triggered immunity
GFP	green fluorescent protein
GDP	guanosine-5'-diphosphate
GTP	guanosine-5'-triphosphate
HCpro	helper component proteinase
HEAT	α-helical repeat motif
HEN1	Hua-enhancer 1
HCm	mutant of HCpro (HCmA, HCmB, HCmC, HCmABC)
HIP1	HCpro-interacting protein 1
HIP2	HCpro-interacting protein 2
HSP	Heat shock protein
HR	hypersensitive response
HVR	highly variable region of HCpro
JA	jasmonic acid
LAR	local acquired resistance

LMV	*Lettuce mosaic virus*
LRR	leucine-rich repeat domain
MAP215	MT-associated protein 215
mRFP	monomeric red fluorescent protein
MT	microtubule
NH_2-	amino-
NB	nucleotide-binding domain
NIa	nuclear inclusion protein a
NIb	nuclear inclusion protein b
Nt	*Nicotiana tabacum*
nt	nucleotide
ORMV	*Oilseed rape mosaic virus*
P-body	RNA processing body
PM	plasma membrane
PP2A	protein phosphatase 2A
PTI	PAMP-triggered immunity
PPV	*Plum pox virus*
PRSV	*Papaya ringspot virus*
PSII	photosystem II
PSbMV	*Pea seed-borne mosaic virus*
pv.	pathovar
PVA	*Potato virus A*
PVAm	mutant of PVA (PVAmA, PVAmB, PVAmC, PVAmABC)
PVY	*Potato virus Y*
PVX	*Potato virus X*
RLK	receptor-like kinase
RLP	receptor-like protein
R protein	resistance protein
SA	salicylic acid
SAR	systemic acquired resistance
siRNA	short interfering RNA
SPR2	SPIRAL2
SP2L	SPIRAL2-LIKE
ssRNA	single-stranded RNA
St	*Solanum tuberosum*
TEV	*Tobacco etch virus*
TMV	*Tobacco mosaic virus*
TuMV	*Turnip mosaic virus*
TOG	tumour overexpressed gene domain
TOR	target of rapamycin
VPg	viral genome-linked protein
wt	wild type
YFP	yellow fluorescent protein
YTHS	yeast two-hybrid system

1 INTRODUCTION

1.1 The targets of pathogens within plant cells

Plant-pathogenic micro-organisms, with few exceptions, occupy the extracellular, apoplastic side of living host cells (biotrophs) or consume the remains of dead cells (necrotrophic pathogens). In contrast, pathogenic viruses are obligatory intracellular parasites that complete their infection cycle within host cells. Both the intracellular and extracellular pathogens have specific targets within plant cells where they induce changes that allow them to fully benefit from the, mainly intracellular, resources of the host. They also have to interact with or within the host cell in order to thwart defence reactions, cope with them, or escape them by spreading to intact tissue. Structural or compositional changes observed in infected cells may indicate that the pathogen has rerouted or recruited host intracellular transport machinery and even altered the placement of host organelles – or, as host subcellular architecture or composition is changed during active defence, that defence responses have been triggered.

The advent of pathogens and of abiotic stresses is perceived via sensors that detect physical pressures or forces at plasma membrane (PM) (Nick 2013) or via chemical receptors located in the cell wall matrix, the PM, cortical cytosol, or inner membranes (Tör et al. 2009). After overcoming a signalling threshold, the initial perception is followed by signal amplification and defence-related processes that involve ligand-induced endocytosis of extracellular receptors, transport of cytoplasmic receptors to new locations, phosphorylation cascades, increased secretion by exocytosis, production of reactive oxygen species, and a general transcriptional reprogramming (Robatzek 2007, Tör et al. 2009).

The pathogens target the plant surveillance system, and the virulence factors and effector molecules of pathogens interact with or regulate various host components. Depending on whether a host component is important for pathogenicity or for virulence, indicate if they are important for the pathogen to infect, or to subdue the plant defence. Some targets of pathogens are even involved in both, as the host factors that pathogens require for infection can in turn be protected by the guarding system of host defence.

Plant cells are surrounded by a cell wall that acts as a physical barrier but also forms a continuum with the PM, its lipid rafts, cytoskeleton and inner membranes (Baluška et al. 2003, Nick 2013). This continuum participates in and is subject to changes from signalling-related endocytosis or defence-related secretion. Plant pathogens actively target the functions of this interface of apoplast and cytoplasm. Effectors of extracellular pathogens enter the cytoplasm by the pathogen's secretory system (e.g., by using the type III secretory machinery of gram-negative bacteria) or hitch-hike the

intake mechanisms of host. Viruses, that multiply inside the cells, apply and reroute parts of the secretory pathway (Wei et al. 2010, Grangeon et al. 2012). Viral infections generally downregulates the expression of genes coding for secreted proteins, cell wall modifying enzymes, and those of secretory pathways (Yang et al. 2007) and measurably reduces secretion to the apoplast (Wei and Wang 2008).

The cytoskeleton of plants consists of actin (microfilaments and vesicular actin) and cortical microtubules (MTs). Actin conducts vesicular and other intracellular traffic and cytoplasmic streaming, while the functions of MTs are more obvious in spatial marking of cellular sites, such as those critical for the cell cycle (marking of the cell plate) or for final position within cells, including anchorage of chloroplasts, directing the locations of Golgi, or deposition of the cellulose synthase complex into PM (Takagi et al. 2009, Vick and Nebenführ 2012). The placement of the cortical MT cytoskeleton in the cytosolic side of the PM, its interactions with the PM and inner membranes, and its fast dynamics all suggest that the MT array might be important for conducting defence signalling. Nevertheless, only one effector of an extracellular phytopathogen is known to directly target MTs, namely the HopZ1a of *Pseudomonas syringae* pv. *syringae* that interacts with MTs and their components, tubulin heterodimers (Lee et al. 2012). HopZ1a is an acetyltransferase that induces dissociation of plant MTs by acetylating tubulin, which is followed by blocked secretion and reduced cell wall associated defences (Lee et al. 2012).

Cell polarization during plant defence involves three commonly observed alterations in subcellular structures, namely movement of cell organelles towards a pathogen contact point, rearrangement of the cytoskeleton, and rearrangement of intracellular membranes for secretion (Schmelzer 2002, Robatzek 2007). Cell death by hypersensitive response (HR) is considered the ultimate defence reaction (Jones and Dangl 2006) and involves multiple subcellular rearrangements, including changes in MTs (Higaki et al. 2011, Smertenko and Franklin-Tong 2011).

This literature review concentrates on plant viruses with positive sense single-stranded RNA ((+)ssRNA) genomes, and on the host factors associated with those viruses, the alterations of subcellular organization during infection and defence, and on the putative role of the MTs in them. Other viruses may share some of the strategies and apply similar mechanisms during their infection cycle, and other pathogens may have acquired similar virulence mechanisms by convergent evolution. Pathogens of other eukaryotes than plants differ in their use of the cytoskeleton due to fundamental differences between their hosts, but some similarities still occur.

1.2 Multiplication of (+)ssRNA viruses in the cell symplast

Viral proteins are translated in the infected host cell. In plants, when an infection has been established in one cell, the virus can move to neighbouring cells via plasmodesmata channels, a cytoplasmic cell-to-cell continuum. Thus, virions and viral proteins stay within the symplast, and may exist in the plant apoplast only rarely and briefly: at the initiation of infection by mechanical or vector-mediated transmission, and during apoplastic unloading in sink leaves of some species (Vuorinen et al. 2011). Hence the viral effectors are produced near their sites of action.

This section reviews the capture and modification of some of the cellular protein complexes and structures upon virus infection, with particular focus on the possible roles of MTs in these alterations, and on the helper component proteinase (HCpro) of potyviruses (genus *Potyvirus*). HCpro is an important and multifunctional viral effector that regulates plant antiviral defences, including RNA silencing. HCpro proteins of many potyviruses are also recognized by plant defence system.

1.2.1 Translation strategies of (+)ssRNA viruses and the polyprotein of Potyviruses

Expression of the genomes of (+)ssRNA viruses is seemingly straightforward, as their genome has mRNA-polarity and serves as a direct template for translation. Nevertheless, host factors need to be recruited for the translation of the initial viral genome and its replicated copies. Viral strategies for translation initiation and viral RNA protection are divergent.

The (+)ssRNA genomes of members of the order *Nidovirales*, most of the *Tymovirales*, and several unassigned families and genera have a poly(A) tract in the 3' end and a cap structure in the 5' end of viral RNA (ICTV 2012). A few unassigned families, including the *Virgaviridae*, have a cap but no poly(A) tract. The cap may be sufficient to direct the viral RNA to the host translation machinery. For instance, fluorescently labelled genomic RNA of *Tobacco mosaic virus* (TMV, genus *Tobamovirus*, family *Virgaviridae*) inoculated in living cells forms endoplasmic reticulum (ER) -associated granules at the initiation of infection in a cap-dependent manner (Christensen et al. 2009).

Members of the largest order of (+)ssRNA viruses, *Picornavirales*, and the unassigned families *Potyviridae, Caliciviridae*, and possibly also *Astroviridae* and genera *Polemovirus* and *Sobemovirus* (ICTV 2012) have a poly(A) tract but lack the cap structure. The cap is replaced by the viral genome-linked protein (VPg) that is covalently attached to the 5' end of the virus genome. In the viruses lacking a cap structure, translation machinery may be recruited by VPg or by RNA structures at internal ribosome entry sites, while the requirement for translation components or factors other than ribosomes varies (Kieft 2008).

Potyviruses (genus *Potyvirus*, family *Potyviridae*) infecting over 140 plant- species share other similarities with the picorna-like supergroup of (+)ssRNA viruses (ICTV 2012). The genomes of potyviruses consist of a single RNA molecule packed into a filamentous flexuous particle. The particle is encapsidated by multiple copies of a coat protein (CP) and a single VPg covalently attached to the 5' end of RNA. The genome is translated as a large polyprotein which is proteolytically processed into 10 proteins and various cleavage intermediates by three viral proteinases (Dougherty and Carrington 1988, Merits et al. 2002, Rajamäki et al. 2004). The first potyviral protein (P1) and the second protein HCpro cleave *in cis* their own carboxyl (C)- termini releasing themselves from the polyprotein. The other cleavage sites are processed by a proteinase domain of nuclear inclusion protein a (NIa) (Rajamäki et al. 2004). Another, shorter open reading frame with a frameshift and an early translational stop codon within the sequence coding for the third protein (P3) is predicted in potyviruses, and a product of such a reading frame, protein P3N-PIPO, has been detected in plants as the 11th potyviral protein (Chung et al. 2008). No viral subgenomic RNA have been reported in plants infected with potyviruses and hence P3N-PIPO must be translated via a ribosomal frameshift from the full-length viral genome, hypothetically as part of a short polyprotein consisting of a normal P1, a normal HCpro and the P3N-PIPO.

The proteins thought to be directly involved in viral replication occur in the large polyprotein consecutively after P3. They are the 6 kilodalton (kDa) protein 1 (6K1); cylindrical inclusion protein (CI) that is an RNA helicase; 6 kDa protein 2 (6K2); NIa that is further processed to VPg and proteinase (NIa-Pro) parts; and nuclear inclusion protein b (NIb) that is an RNA-dependent RNA polymerase, the viral replicase. This sequence corresponds to the typical core replicative module of picorna-like viruses with the order helicase-proteinase-polymerase (reviewed by Le Gall et al. 2008). The last protein of the polyprotein is the CP, in a genomic position different from that of members of the *Picornavirales*.

1.2.2 Competition for translation factors by viruses and regulation of translation in stress responses

Eukaryotic cells sort, regulate, store, translate and degrade mRNA molecules by their secondary structures. Eukaryotic translation initiation factor (eIF) 4E (eIF4E) is a cap-binding protein that, together with scaffold protein eIF4G and helicase protein eIF4A, forms a translation initiation complex eIF4F. In plants, an alternative complex, eIF(iso)4F, is formed by isoforms of the eIF4E and eIF4G proteins. eIF(iso)4E differs from eIF4E in its affinity to different RNA 5´translation leader structures and a higher affinity to the cap structure (Gallie and Browning 2001).

The importance of the eIF4E and eIF(iso)4E as essential host factors for potyvirus infection is reflected by the multiplicity of recessive resistance

traits that are based on alleles of these genes in economically important crop species (reviewed in Wang and Krishnaswamy 2012). These resistances are effective against potyviruses, and both broad- and narrow-spectrum resistances are known. Interaction of NIa, and particularly its VPg part, with either of these host factors is required for successful infection by some potyviruses (Wittman et al. 1997, Léonard et al. 2000, Schaad et al. 2000, Duprat et al. 2002, Lellis et al. 2002, Ruffel et al. 2002 Kang et al. 2005). The interaction of VPg and eIF4E proteins may be related to enhancement of viral translation, as expression of VPg in plants enhances the translation of RNA with the potyviral 5' untranslated region and decreases the cap-dependent translation of mRNA (Miyoshi et al. 2008, Eskelin et al. 2011). In addition to VPg, CI of *Lettuce mosaic virus* (LMV, genus *Potyvirus*) also interacts with eIF4E (Tavert-Roudet et al. 2012).

Hypotheses about the role of recruitment of eIF4E proteins by viruses speculate on downregulation of host protein synthesis, and on the enhanced synthesis of virus proteins, on confiscation of necessary translation factors, and on protection of viral RNAs (e.g., Lellis et al. 2002). The eIF4E-related resistances not only restrict virus multiplication, but also systemic movement of some potyviruses, by an unknown mechanism. eIF(iso)4G genes may also have a role in translational regulation under different stresses, as indicated by the various defects observed in *Arabidopsis* (thale cress, *Arabidopsis thaliana* (L.) Heynh) knockout plants: chlorosis, defects in growth, development and photosynthesis, and altered responses to stresses (Lellis et al. 2010). Still, the basic translational activity of the eIF(iso)4G knockout plants appears unimpaired, consistent with the requirement of eIF(iso)4G proteins mainly for translation or other regulation of specific mRNAs (Lellis et al. 2010).

Cellular proteins that interact with eIF4E via a 4E binding motif (4EBD) sterically inhibit cap-dependent translation initiation of some mRNAs (Richter and Sonenberg 2005, Rhoads 2009). *Encephalomyocarditis virus* (genus *Cardiovirus*, family *Picornaviridae*) protein 2A, involved in translational shut-off of host proteins, interacts with human eIF4E via a 4EBD motif (Groppo et al. 2011), but 4EBD motifs are not known in other viruses.

The scaffold protein eIF4G increases the affinity of eIF4E to the VPg of potyviruses *in vitro* (Grzela et al. 2006), and the VPg of *Rice yellow mottle virus* (genus *Sobemovirus*) directly interacts with eIF(iso)4G (Hébrard et al. 2010). Viruses of families that lack VPg and the poly(A) tract and have either uncapped (*Tombusviridae*) or capped (*Bromoviridae*) genomes, are also dependent on a suitable form of eIF4G (Yoshii et al. 2004). Transcriptional changes induced during virus infection include upregulation of translation- and folding-associated transcripts including genes coding for ribosomal and chaperonic heat shock proteins (HSP) (Whitham et al. 2003, Yang et al. 2007). The chaperones in particular are also induced during signalling for biotic stresses and participate in receptor biogenesis (Caplan et al. 2009).

Eukaryotic mRNAs can be stored on MT-associated granules (Chernov et al. 2008). Maskin, an interaction partner of MT-associated protein 215 (MAP215) in the amphibian *Xenopus laevis* (Daudin, 1802), interacts with eIF4E, regulates its localization, and functions in the translation of stored mRNA (Barnard et al. 2005). The prevalence of plant MTs in translational control is little studied, but RNA-processing bodies called P-bodies transiently associate with MTs also in plants (Hamada et al. 2012). Maize eIF4G may be an MT-associated protein as it binds MTs *in vitro*, either alone or in complex with eIF4E (Bokros et al. 1995). The *Arabidopsis* knockout line of eIF(iso)4G genes is oversensitive to salinity and unresponsive to heat shock (Lellis et al. 2010), both being stresses that invoke MT-dependent signalling in plants (Nick 2013).

1.2.3 Compartmentalization and structural alterations of endomembranes in virus infection

RNA viruses replicate on ER and other membranes in animal and plant hosts (Salonen et al. 2005, Miller and Krijnse-Locker 2008, Verchot 2011), indicating that membrane-associated functions are important for viruses. Infection by plant viruses induces morphological changes in membranes (Schaad et al. 1997, Reichel and Beachy 1998) and many transiently or transgenically expressed viral proteins alter membranes in plants (Wei et al. 2008, Shand et al. 2009, Zheng et al. 2011) or *in vitro* (Rantalainen et al. 2009).

During potyvirus infection, membraneous vesicles are induced at ER-exit sites (ERES) by the viral protein 6K2 and transported to the proximity of chloroplasts by COPII- and actin-dependent transport (Wei and Wang 2008, Wei et al. 2010). The viral RNA and translation- or replication-associated proteins are detected as colocalized with these structures.

The newly synthesized viral genomes are transported from the replication sites to the cell periphery as ribonucleoprotein complexes that then move to the next cells via the membrane-lined plasmodesmata (Ueki and Citovsky 2011). Viral movement proteins are often associated with membranes and are capable to enlarge plasmodesmata, allowing passage of viral particles or ribonucleoprotein complexes. For instance, TMV movement protein (MP) reorganizes ER, an ability correlated with its MT-localization (Reichel and Beachy 1998, Ferralli et al. 2006). In the cells that are at the infection front, the ER-embedded replication-associated complexes of TMV might be translocated at the cell cortex towards plasmodesmata via an MT-nucleation dependent mechanism (Ouko et al. 2010). Furthermore, some potyviral replication vesicles have been observed in cell peripheries (Grangeon et al. 2012), but the functions and fine-scale localizations of that subpopulation have not been studied.

Reorganization of ER and consequent endomembrane traffic should have a critical role in virus accumulation (Miller and Krijnse-Locker 2008).

Intracellular and intercellular movements of the viral RNAs or components are an intrinsic part of viral replication, accumulation and movement strategies (Harries et al. 2010), making their experimental separation a challenge.

1.2.4 Viral inclusions and protein bodies

Aggregation of viral proteins occurs commonly and the MT-dependent formation of perinuclear aggresomes is important for animal virus multiplication (reviewed by Wileman 2006). Similar mechanisms may function in plants in the formation and concentration of viral replication complexes and their enlargement into viral factories for production of viral progeny, or in the formation of viral movement complexes (Niehl et al. 2013). Protein bodies or small cortical foci have been suggested to have roles in early stages of virus replication or plasmodesmata targeting and cell-to-cell movement (e.g., Christensen et al. 2009, Shemyakina et al. 2011, Cho et al. 2012, Niehl et al. 2013) while the larger cortical MT-associated inclusions may be deployed in transmission of viruses (Martinière et al. 2009) and the perinuclear aggregates or inclusions as parts of virus factories (Wei et al. 2010, Grangeon et al. 2012, Niehl et al. 2013).

Several potyviral proteins accumulate to inclusions in infected cells, including HCpro that forms amorphic inclusions in the cytosol (reviewed by Purcifull and Hiebert 1992, Rajamäki et al. 2004). These have been visualized in plant cells using fluorescence tags (Dielen et al. 2011, Tatineni et al. 2011, Zheng et al. 2011) but their roles in the infection cycle and the mechanism of their formation are not known yet. For instance, HCpro of LMV forms aggregates near the nucleus during virus infection (Dielen et al. 2011), and the transiently expressed HCpro of *Turnip mosaic virus* (TuMV) forms granules that are potentially ER-associated (Zheng et al. 2011). HCpro of LMV and *Potato virus Y* (PVY) interact with the subunits of the proteasome and interfere with its functions (Ballut et al. 2005, Jin et al. 2007a), which could explain why these aggregates are not degraded.

Studies of ER- and MT-associated inclusions of viral proteins suggest that neither of these is a definite final location of the viral proteins. Puncture of a cell by an aphid stylet dissolves and distributes the MT-associated bodies formed by P2 and P3 proteins of CaMV, which presumably enhances virus transmission (Martinière et al. 2009, 2013). Allocation of MP of TMV between ER and cytosol is regulated by a chaperone that mobilizes MP from ER-associated inclusions, and the soluble proteins increasingly associates with MTs at later stages of infection (Niehl et al. 2012).

1.2.5 Chloroplast alterations and cell polarization

Specific alterations in chloroplast morphology and organization have been observed during potyvirus infection. Morphology defects, including

extensions of the outer membrane into lobed or pocket structures, are induced during accumulation of replication vesicles on the peripheries of chloroplasts, possibly attached on the chloroplast envelope (Wei et al. 2010). Chloroplast stromules or structural alterations are also induced during infection by the flavum strain of TMV (Lehto et al. 2003, Caplan et al. 2008). Caplan et al. (2008) observed an altered location of NRIP1, a chloroplast protein guarded by resistance protein N that triggers HR to TMV, which they proposed to belong to the proteins that are exported from the chloroplast upon virus infection.

In the final stages of potyvirus infection, chloroplasts agglomerate to the perinuclear region, surrounding putative viral factories that are formed of amalgamated ER and Golgi membranes and viral and host proteins (Wei et al. 2010, Grangeon et al. 2012). The chloroplasts may provide a protected and energy-rich environment for virus replication and assembly.

MTs participate in stable spatial positioning of chloroplasts after actin-mediated light-guided movements (Takagi et al. 2009). It is possible that MTs may directly participate in the chloroplast transport, for example, in regenerative cells (Cai and Cresti 2010). Furthermore, transient chloroplast-MT-peroxisome interactions are indicated in the development of proplastids into chloroplasts in cotyledons (Albrecht et al. 2010). Morphological defects, such as formation of bi-lobed chloroplasts and stromule-like structures, have been observed upon treatment with MT-disrupting chemicals, suggesting that MTs may be required for chloroplast development and protein exchange between cell organelles (Albrecht et al. 2010).

1.3 Plant defence and the molecular basis of symptoms in virus infection

1.3.1 Plant innate immunity and antiviral defence

Understanding of the constant evolution of the heritable immunity system in plants is fundamentally based on the model presented by Jones and Dangl (2006), and their model can be adjusted to encompass antiviral defence (Mandadi and Scholthof 2013). Jones and Dangl separate plant immunity into two defence response classes based on the evolutionary history and commonality of the defence-triggering signals. The two categories, pathogen-triggered immunity (PTI) and effector-triggered immunity (ETI), differ in the sense that PTI recognizes molecular patterns commonly associated with a wide variety of pathogens rather than individual pathogens, while ETI targets effectors that specific pathogens produce in order to overcome PTI. ETI is based on gene-for-gene recognition mediated by the so-called resistance (R) proteins that directly or indirectly recognize pathogen effectors or their action (Van der Biezen and Jones 1998).

In defense against extracellular pathogens, PTI is generally dependent on extracellular or PM-associated pattern receptors, the receptor-like proteins (RLPs) and receptor-like kinases (RLKs), while ETI is triggered mainly by intracellular receptors, namely the R proteins (Tör et al. 2009). R proteins have a nucleotide-binding site and leucine-rich repeat (NB-LRR) domain structure, and they guard the cellular targets vulnerable to pathogen action: pattern receptors, their downstream signalling components, or the host factors that a pathogen requires for successful infection. R proteins and pattern receptors are found in multiple locations within the cell, but their spatial distribution and its role in signalling are not fully understood.

Infection by compatible viruses elicits transcription of genes related to defence and salicylic acid (SA), ethylene (ET) or jasmonic acid (JA) signalling in susceptible plants, but these responses either do not lead to functional defence or may even enhance viral infection (Whitham et al. 2003, Love et al. 2005). Particular pattern receptors in defence against plant viruses have not yet been identified, but RNA silencing might be a form of PTI (Zvereva and Pooggin 2012).

The local responses categorized as ETI and PTI differ widely in magnitude and specificity and their signalling cascades share components (Thomma et al. 2011). ETI is commonly followed by a local programmed cell death, HR, that is effective against biotrophic pathogens. Defences in unchallenged parts of plants, including systemic acquired resistance (SAR), can be induced both by ETI and PTI signalling (Mishina and Zeier 2007). For example, *Arabidopsis* CRT1 (compromised for recognition of *Turnip crinkle virus* 1) is located in the endosomes and nucleus, is required for ETI, PTI and SAR signalling, and interacts with several R proteins and with the pattern receptor FLS2 (Kang et al. 2008, 2010, 2012).

Resistances based on pathogen recognition are usually dominant traits and lead to active defence responses. The first cloned antiviral R genes were from the solanaceous plants: the *N* gene introduced to common tobacco (*Nicotiana tabacum* L.) for resistance against TMV, and the *Rx1* gene of potato (*Solanum tuberosum* L.) against *Potato virus X* (PVX, genus *Potexvirus*) (Whitham et al. 1994, Bendahmane et al. 1999). Interestingly, defences mediated by R proteins also provoke translational inhibition that is specific to structures of the parasitic RNA molecule: in addition to HR-related cell death, *N*-mediated resistance inhibits translation of the subgenomic RNA of PVX (Bhattacharjee et al. 2009). Similarly, the inhibition of virus replication and the induction of cell death by Rx1 were shown to be separate functions (Bendahmane et al. 1999).

Some dominant antiviral traits restrict particular processes of the viral infection cycle without triggering immunity signalling or cell death. Multiplication of *Tomato mosaic virus* (genus *Tobamovirus*) in tomato (*Solanum lycopersicum* Lam.) is inhibited by a membrane protein, Tm-1, that inhibits replication via direct interaction with viral replicase (Ishibashi et al. 2007). On the other hand, restriction of long-distance movement of

Tobacco etch virus (TEV) and some other potyviruses in *Arabidopsis* does not include restriction of virus accumulation or local movement (Whitham et al. 2000). This trait, resistance to TEV movement (RTM), involves loci (*RTM1*, *RTM2* and *RTM3*) that code for three unrelated proteins, which are unrelated also to pattern receptors or R proteins (Chisholm et al. 2000, Whitham et al. 2000, Cosson et al. 2010).

Recessive resistance traits, like those based on eIF4E, are usually due to lack of compatible host factors. Obligatory biotrophs and intracellular parasites are more likely to be restricted by this passive form of resistance. This scenario fits into the relative abundance of recessive traits for virus resistance (Diaz-Pendon et al. 2004). Recessive resistances against plant viruses are associated with lack of replication, accumulation, or systemic movement. They can also appear as quantitative traits, which only partially inhibit virus multiplication (Nicaise et al. 2003). Potentially, recessive resistance may also be accompanied by elicitation of active defence (Gonzalez-Ibeas et al. 2012), for example, if the recessive resistance is based on a lack of a host factor that a virus would require to counteract defence.

1.3.2 Durability of dominant and recessive resistance traits is determined by fitness costs and genetic background

Viral fitness, defined as "the capacity of a virus to produce infectious progeny in a given environment" (Domingo and Holland 1997), is the product of replication rate and competitiveness. Virulence, the measure of pathogenicity (Hunt et al. 1994), that is, the amount of disease, can be measured as the fitness cost imposed by the virus on a plant. Evolutionary population dynamic studies on RNA viruses have suggested (though the molecular mechanisms are unknown) that viral fitness is often poorly correlated with virulence and even that attenuation of symptoms is a likely outcome when there is a variation in virulence (Carrasco et al. 2007, Delgado-Eckert et al. 2011).

Success in avoiding host recognition can be associated with a fitness cost to a virus. Examples of this are PVY overcoming the R gene (*Pvr4*) mediated resistance in pepper (Janzac et al. 2010) and a pathotype of TuMV overcoming a polygenic resistance in rapeseed (*Brassica napus* L.) based on known and putative R genes (Jenner et al. 2002). Importantly, the fitness costs severely deter the resistance-breaking isolates, thus increasing the durability of resistance trait in the field – a matter that should be considered in breeding programs (Jenner et al. 2002). Combining dominant R genes into polygenic resistance is important for the durability of resistance (Palloix et al. 2009). This is mainly because a background with multiple resistance traits reduces the accumulation of an infectious virus, but also because multiple mutations are needed to increase virulence so that the pathogen variants that overcome any of the separate resistances are only slowly selected (Quenouille et al. 2013).

Two linked loci, *va* and *va2*, provide recessive resistance to systemic movement of PVY and TVMV in tobacco (Acosta-Leal and Xiong 2008). The ability to overcome *va*-associated resistance in both viruses is determined by VPg (Nicolas et al. 1997, Masuta et al. 1999). The widespread use of *va* in tobacco cultivars without effective dominant R genes has contributed to the prevalence of resistance-breaking aggressive (necrotic) strains of PVY in the field (Lacroix et al. 2010, 2011). A more detailed analysis discovered that the durability of resistance to PVY is improved if the two loci were present, which indicated that they together restrict virus multiplication, delaying appearance of resistance-breaking isolates (Acosta-Leal and Xiong 2008). Hence, resistances that reduce virus amplification may prove useful, though a continued use of recessive resistance traits alone can be problematic.

Any viral components that are under strong stabilizing selection are promising targets for resistance, as mutations in those are more likely to be costly for the viruses. However the risks of resistance breaking are unknown. To understand the evolution of viruses in agricultural systems, it is critical to understand the distributions of virulence and fitness within virus populations and different host plants, but also the genetic background of the resistant plants and the molecular mechanisms responsible for resistance and susceptibility. Information at the molecular level, like the direct interactions of viral components with host factors and how those are determined, may help to explain the different selection pressures and fitness costs imposed by different resistance mechanisms on viruses.

1.3.3 RNA silencing suppression and viral symptoms

RNA silencing as a defence mechanism targets foreign nucleic acids, including viral genomes. RNA silencing is initiated by double-stranded RNA (dsRNA) produced by the viral replicative complexes or locally folded hairpin structures of viral RNAs. The dsRNA is cleaved and unwound, and a short ssRNA product, a primary short interfering RNA (siRNA), guide this complex to any homologous long RNA targets. These cleavage products can be further amplified into new dsRNAs by host-encoded RdRp proteins, which can then be diced into secondary siRNA species. Hence, after an initiation phase, the silencing signal is amplified and the transition mechanisms produce secondary siRNA species that efficiently target their homologous RNA molecules, expanding the range of the recognized and targeted sequences (Baulcombe et al. 2007). The accumulating siRNA is protected from degradation by methylation (Ebhardt et al. 2005). As the RNA silencing machinery is triggered by the replicative (ds) structures of viral RNAs, it can be categorized, despite its adaptable nature and sequence specificity, essentially as PTI (Bilgin et al. 2003, Bowie and Unterholzner 2008). The components of silencing pathway are upregulated during defence signalling, tighty linking RNA silencing to innate immunity (Zvereva and Pooggin 2012).

RNA silencing is also involved in developmental regulation via endogenous siRNA and microRNA (miRNA). Viruses have evolved to suppress this defence pathway at various points, or to shield viral RNA from recognition, which also affects the endogenous siRNA and miRNA signalling. The potyviral silencing suppressor HCpro interferes with endogenous miRNA pathways (Mallory et al. 2002, Dunoyer et al. 2004) and also interacts with an ET-dependent transcription factor, RAV2, that is required for some of the developmental alterations and transcriptional reprogramming in *Arabidopsis* (Endres et al. 2010). Interference with RNA silencing and deployment of RAV2 together may explain many of the developmental or morphological perturbations and viral-like symptoms in HCpro transgenic plants (Siddiqui et al. 2008, Endres et al. 2010, Soitamo et al. 2011). While stress and developmental signalling are integrated, it is possible that HCpro regulates one in order to affect the other.

1.3.4 Necrotic symptoms triggered by R protein-mediated recognition of silencing suppressors

The dominant or R-gene based virus resistance triggers three types of responses: cell death, restriction of virus movement, and inhibition of virus multiplication. The various cell death phenotypes involve extreme resistance without apparent cell death, single cell death or local HR (Bendahmane et al. 1999), as well as a spreading or a systemic necrosis, the induction of which is not well understood but may involve defence misregulation (Mandadi and Scholthof 2013). The genetic background of host plant affects the manifestation of the R-gene associated cell death, as has been shown with potyviruses (e.g., Palloix et al. 2009, Singh et al. 2008, Vuorinen et al. 2010). Some general downstream signalling pathways of R proteins have been described based on the structural families of these proteins. However, the majority of R proteins remain uncharacterized (e.g., Lewis et al. 2010).

R protein mediated antiviral defence often involves recognition of viral effectors, including silencing suppressors. Strong viral silencing suppressors, like HCpro, induce necrotic responses if they are expressed from heterologous virus vectors (Pruss et al. 1997, Brigneti et al. 1998). These responses share similarities to HR, indicating that recognition of a mislocated or misregulated silencing suppressor is the trigger of necrosis (Pruss et al. 1997).

The viral determinants that induce necrosis can be mapped by constructing recombinants of non-necrotic and necrotic viral isolates or strains and studying their pathotypes. Such studies have identified HCpro, either alone or together with other viral proteins, as a possible HR-inducing factor. For instance, HCpro of PVY-O strain induces HR in a potato cultivar with the Ny_{tbr} resistance gene (Moury et al. 2011, Tian and Valkonen 2013). In the *Soybean mosaic virus* (SMV) – soya bean (*Glycine max* (L.) Merr.) pathosystems, coadaptation of HCpro and P3 is enforced, as the *Rsv1* locus

containing several linked R genes recognizes both P3 and HCpro (Eggenberger et al. 2008, Hajimorad et al. 2008, Chowda-Reddy et al. 2011, Wen et al. 2013). A tolerant lettuce (*Lactuca sativa* L.) cultivar shows only mosaic symptoms in infection by LMV-O, but necrotic symptom are induced when the region coding for HCpro is swapped for that of an aggressive strain of LMV (Redondo et al. 2001). Veinal necrosis is determined in PVY-N infected tobacco by two amino acids in HCpro and by other changes in P3 (Tribodet et al. 2005), but also by other regions of the PVY genome (Faurez et al. 2012).

Natural attenuation mutants of potyviruses *Zucchini yellow mosaic virus* and *Clover yellow vein virus* (ClYVV) are used for cross-protection against severe strains of the same viruses. They have mutations within HCpro that interfere with RNA silencing suppression activity or specifically with miRNA binding by the silencing suppressor protein (Shiboleth et al. 2007, Yambao et al. 2007). Altough the viral accumulation is not significantly altered, the host plants show fewer symptoms or are otherwise more tolerant to infection with attenuated strains, as compared to the normal strains which cause severe necrotic symptoms, reducing the crop yeild and its quality and value. It is unknown whether the reduction of necrotic symptoms is caused by the reduction of silencing suppression, or by the avoidance of host recognition.

1.3.5 The influence of viral diseases on plant growth

Various cellular perturbations induced by viruses may, at a macroscopic level, be manifested as non-necrotic viral symptoms. Viral symptoms should not be seen only as a virulence-related phenomenon. The efficiency of transmission is an important component of viral fitness at the ecosystem level, and some viral symptoms, such as those affecting the colour of the canopy may be beneficial for vector-mediated transmission (Wargo and Kurath 2012).

The stunting and loss of shade-avoidance associated with potyvirus infection indicate a reduced phenotypic plasticity of the infected plants (Bedhomme and Elena 2011). The molecular mechanism is unknown, but for example the HSPs that are affected by viral infections may be important for cellular homeostasis and phenotypic plasticity (Krtková et al. 2012). Dwarfing symptoms in diseased plants have been attributed to direct interference with gibberellin synthesis (Zhu et al. 2005), and developmental symptoms to direct viral interactions with components of ET and auxin signalling pathways (Padmanabhan et al. 2005, Endres et al. 2010).

The presence of chlorosis in plants infected by potyviruses correlate with high viral titers, redox changes and downregulated transcription of both nuclear and chloroplast genes for photosynthesis proteins (Rodríguez et al. 2012). Infection by the tobamovirus TMV induces accumulation of starch in tobacco chloroplasts, while the flavum strain of TMV that causes distinct yellow-mosaic and chlorotic symptoms induces distortion of chloroplast

morphology (Lehto et al. 2003). Infection by the flavum strain of TMV reduces the amounts of photosystem II (PSII) proteins, involving translational control of a chloroplast-encoded protein of the PSII complex (Lehto et al. 2003). Furthermore, potyviral proteins interact with chloroplast-targeted proteins: NIa-Pro of *Papaya ringspot virus* (PRSV) interacts with a chloroplast-targeted methionine sulfoxide reductase (Gao et al. 2012), HCpro of *Sugar cane mosaic virus* interacts with transit peptide of a chloroplast-targeted ferredoxin (Cheng et al. 2008) and HCpro of PVY with a putative chloroplast division-related protein (Jin et al. 2007b). These interactions indicate that viruses can interfere with chloroplast targeting of proteins and chloroplast development. In addition to regulation of the energy metabolism, these interactions may be needed for alleviation of chloroplast-associated defences.

1.4 The MT cytoskeleton in subcellular organization and defence signalling

1.4.1 Structure and organization of MTs

Microtubules distribute the nuclear genetic material during meiosis and mitosis, and mark the position of new cell plates formed in cell division. At interphase, the MTs of yeast and most animal cell types form MT bundles that originate from a single perinuclear MT-organization centre (MTOC) and extend radially across the cell to mark polarity and to function as the main routes for intracellular transport (Höög et al. 2007). The viruses of animal cells use MTs and MT motor proteins for the transport of virions or viral components between the perinuclear regions and cell peripheries (Leopold and Pfister 2006, Radtke et al. 2006, Ward 2011). Transport on MTs facilitates several steps of the infection cycle, including the initiation of infection, the accumulation of viral proteins, and replication, assembly and subsequent egress of the progeny virus (Dodding and Way 2011).

The plant kingdom deploys two special MT structures during cell division: a transient preprophase band in the cell cortex that marks the position of the future cell plate, and a phragmoplast structure with MTs at telophase that directs the formation of the new cell plate and cell walls. The discovery of cortical MT arrays as the major form of microtubule localization of plant interphase cells of (Ledbetter and Porter 1963) indicated a fundamental difference in and a unique organization of the MTs in the plant kingdom. The cortical MT array consists of coaligned or bundled MTs that undergo mosaic-wise rotary movements and form highly organized parallel arrays, especially in expanding cells (Cyr and Palevitz 1995, Chan et al. 2007, Ehrhardt 2008).

The tubular structure of MTs is a result of polymerisation of heterodimers composed of alpha (α) and beta (β) tubulin (**Fig. 1**). The heterodimers assemble into protofilaments that further form a sheet-like structure,

gradually closing to a hollow tubular structure, a microtubule. The polymerization of tubulin heterodimers into MT occurs *in vitro* without need of other components but is greatly increased by MT-associated proteins *in vivo*. The net growth or shrinkage of an individual MT is an outcome of dynamic instability, a process composed of a constant switching between phases of growth and shrinkage (Mitchison and Kirschner 1984).

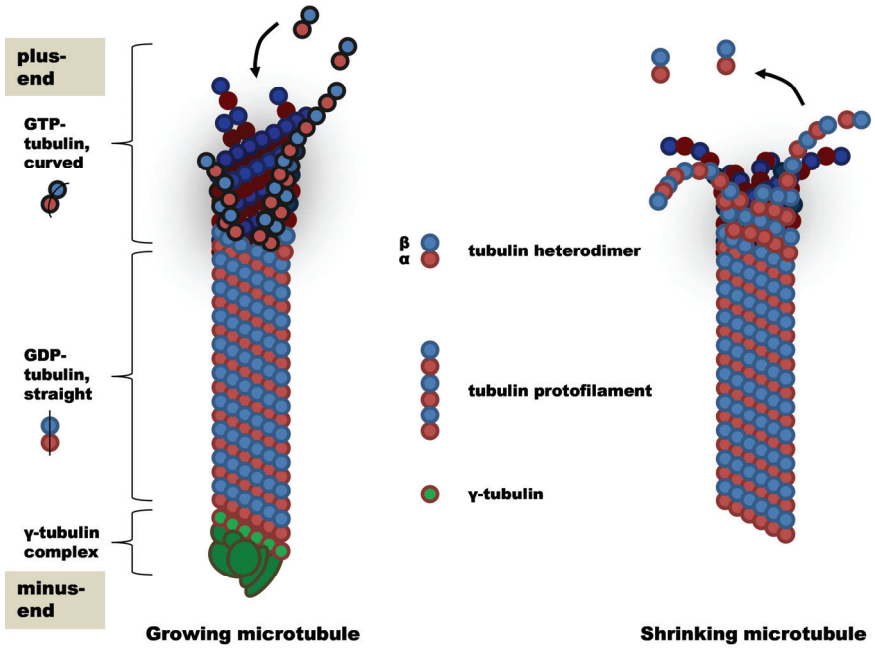

Figure 1 Schematic drawing of the microtubule (MT) structure. The MT consists of α-β tubulin heterodimers. The minus-end of a newly formed MT is transiently capped by γ-tubulin and γ-tubulin complex. The (+)-end of MT is more dynamic and rapidly polymerizes or shrinks between pause events. GTP and GDP, guanosine-5'-triphosphate and guanosine-5'-diphosphate, respectively, indicate the tubulin phosphorylation state. The regulation of MT dynamics by MT-associated proteins is not illustrated. Adapted from Akhmanova and Steinmetz (2008).

MT-associated proteins can directly interact with tubulin. In this thesis, the term "MT-associated protein" is used whether the protein is observed along the MTs or at their ends. MT-associated proteins affect the general organization (like bundling or branching) and dynamics of the MT cytoskeleton via interactions with soluble tubulin, the MT lattice, and other MT-associated proteins. MT-associated proteins with direct activity in MT polymerization or severing are often located at the MT (+)-ends.

1.4.2 Modularity and repeat elements in MT-associated proteins

Genomic duplication events lead not only to duplicated genomes, genomic regions or genes, but also to duplicated internal parts of genes, creating regular structures. Modularity and modular scaffold proteins are important in complex systems with multiple alternative protein interacting components (Bhattacharyya et al. 2006). MTs, being polymers, are self-associating repeated structures. MT-associated proteins are particularly rich in modular interaction domains and repeat elements (Akhmanova and Steinmetz 2008, Steinmetz and Akhmanova 2008). These are, in addition to tubulin interactions, involved in mutual interactions and self-interactions that are commonly required for tracking MT (+)-ends (Slep and Vale 2007). Many MT end- and MT lattice-associated proteins self-interact and may locate on MTs as dimers or oligomers (Li et al. 2007, Stoppin-Mellet et al. 2007, Blake-Hodek 2010, Motose et al. 2011).

At the structural level, repeat elements in proteins can be classified according to the secondary structure elements (α-helices and β-sheets) and whether the repeat arrays are organized into open or closed conformations (Branden and Tooze 1999). Tandem repeats, composed of helical pseudo-repeats of helix-turn-helix structures, are classified into several subfamilies. Andrade and Bork (1995) discovered and named HEAT-motifs according to the four proteins where they were found: huntingtin, elongation factor 3 (EF3), A-subunit (65kDa) of protein phosphatase 2A (PP2A), and lipid kinase TOR (target of rapamycin). HEAT and related armadillo (ARM) motifs are formed by units of two or three α-helices, respectively, and are found in at least 0.2% of eukaryotic proteins (Andrade et al. 2001), including several MT-associated proteins (Slep and Vale 2007). An important domain in MT polymerases, the tumour overexpressed gene (TOG) domain, is formed of consequtive HEAT-repeats. The TOG domains are present as multiple copies in eukaryotic MT-associated protein families MAP215 (Andrade et al. 2001) and in the related cytoplasmic linker protein 170 associated proteins (CLASP) (Al-Bassam et al. 2010). The HEAT repeats are also prevalent in proteins present in alternative complexes, such as those functioning in signalling, translation or transport processes (Andrade and Bork 1995). For example, a human scaffold protein of translation initiation complex, eIF4GII, has five HEAT repeats (Marcotrigiano et al. 2001).

Two common features of both HEAT and ARM repeat motifs are the connection of single repeat units by short turns and their adjacence in the primary sequence. The solved structures of arrays consisting of HEAT or ARM repeats have been described as “handed superhelix” or “elongated α-solenoid”, and as they are flexible or elastic, they may wrap around their interaction partners (Conti et al. 2006, Kappel et al. 2010). Such an extended solenoid structure has a larger surface area than a purely globular protein, which potentiates it to function as a scaffold or interaction platform.

Coiled-coil (CC) is a third type of motif common in MT-associated and centrosomal proteins (Akhmanova and Steinmetz 2008, Zizlsperger and

Keating 2010). CC is also a helical motif, and requires a tight interaction between the participating helices, though the helices may be apart in the primary sequence or even located in separate peptide chains (Moutevelis and Woolfson 2009). While the HEAT and ARM repeats are flexible, CC motifs form rod-like rigid arrays.

1.4.3 Conserved and plant-specific functions of cortical MTs in cellular architecture and signalling

Plant cortical MTs are attached to the PM at many points (Cai et al. 2005, Barton et al. 2008, Sainsbury et al. 2008). There are several possible MT nucleation centres as the γ-tubulin ring-complex required for MT nucleation occurs along the MT and in MT intersections (Chan et al. 2003, Dryková et al. 2003, Pastuglia and Bouchez 2007). New MTs in *Arabidopsis* indeed arise along existing ones, or from their crossings, resulting in acentriolar organization (Murata et al. 2005, Chan et al. 2009). In scanning-electron microscope images, the MTs closest to the PM appear the most stable, and protein complexes bridge them to the membrane, while a more dynamic subpopulation exists deeper in the cytoplasm (Barton et al. 2008). Electron microscope studies have also found MT-associated vesicles that are involved in cell wall formation or other secretion, or in endocytic traffic (Crowell et al. 2009, Gutierrez et al. 2009, Kaneda et al. 2010) (**Fig. 2**).

Cortical MTs control cell division and expansion, and thus cell differentiation and morphogenesis. Oversensitivity to MT-affecting pharmacological agents such as oryzalin, colchicine or taxol, and a spiral or helical growth habit, are phenotypes indicating mutations in genes important for MT functions (Furutani et al. 2000, Hashimoto 2002). Various mutations in genes for α, β, or γ-tubulin or MT-associated proteins induce such spiral growth (Hashimoto 2002, Buschmann et al. 2004, Sedbrook et al. 2004, Shoji et al. 2004, Ishida et al. 2007, Nakamura and Hashimoto 2009, Ruggenthaler et al. 2009).

The reception of physical signals by the cortical MT array has been recently discussed by Nick (2013). MTs are self-supportive, stiff, hollow cylinders and so they transmit compression forces, but in plants they also have acquired a role in perceiving cell integrity (Nick 2013). The minute tensions at PM induced by external forces, osmotic changes or changes in membrane fluidity upon cold stress (Bisgrove et al. 2008, Hoefle et al. 2011) are conveyed to chemical signals at MTs. The perception of and acclimation to abiotic stresses is associated with transient losses of cortical MTs (Naoi and Hashimoto 2004, Komis et al. 2011), which in salt-stress is proteasome-dependent (Wang et al. 2011).

The architecture or dynamics of MTs is altered upon perception of biotic signals in interactions with viruses, nematodes, pathogenic or symbiotic bacteria or fungi, and upon application of some effectors and toxins, but the initiation and role of these changes are yet unclear (Hardham 2013). For

example, treatment with the bacterial elicitor harpin disrupts MTs in cells of tobacco and grapevine (*Vitis vinifera* L.) (Qiao et al. 2010, Guan et al. 2013), but no direct interactions between harpin and MTs were demonstrated. Harpin homo-oligomerizes to make pores that compromise host PM integrity (Haapalainen et al. 2011), so the disruption of MTs in this case may be a general response in induction of cell death.

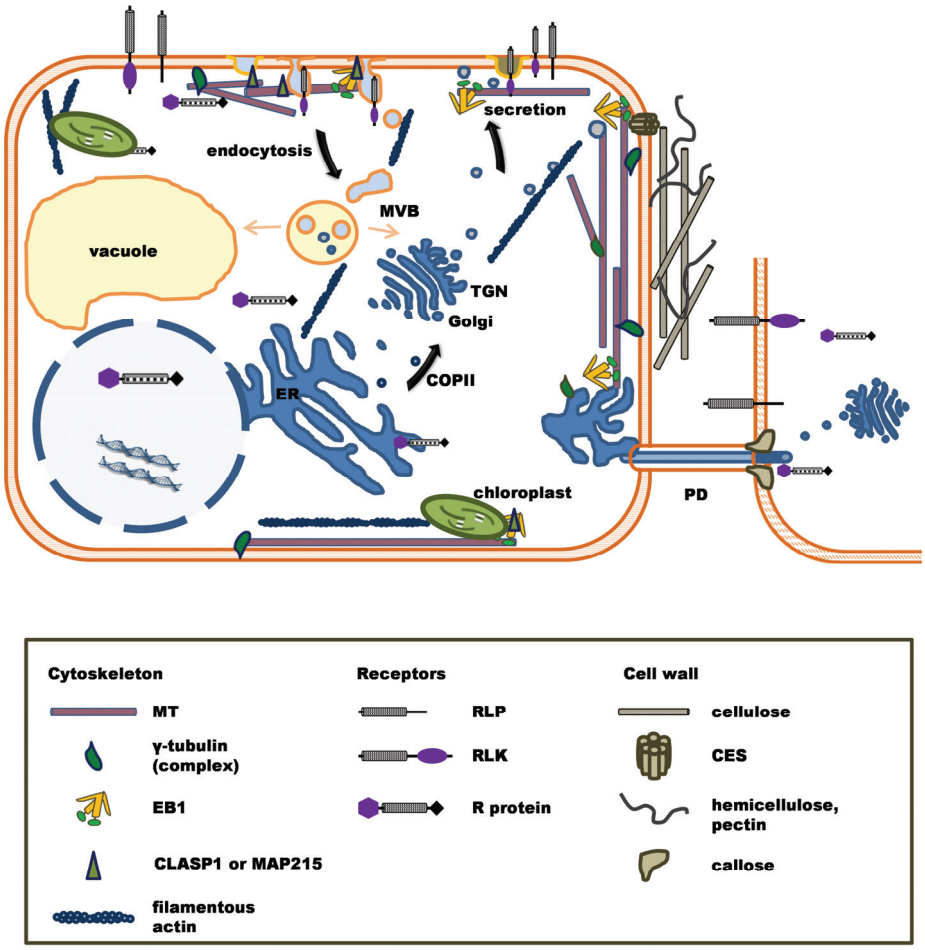

Figure 2 Schematic drawing of cortical MTs in a plant cell and relations of the MTs to the plasma membrane, endomembranes, secretion and endocytosis, and signalling. CES, cellulose synthase complex; COPII, vesicular coat protein II complex; CLASP1, cytoskeletal linker protein 170 associated protein; EB1, end-binding 1; ER, endoplasmic reticulum; MAP215, MT-associated protein 215; MVB, multivesicular body; MT, microtubule; PD, plasmodesma; R protein, resistance protein; RLK, receptor-like kinase; RLP, receptor-like protein. Cytoskeletal motor proteins are not shown.

The dynamic instability of MTs is regulated in cell division, in directing cell expansion, and in sensing of gravity and extra- and intracellular signals (Bisgrove et al. 2008, Shi et al. 2009, Qiao et al. 2010, Hoefle et al. 2011, Yao et al. 2011). Chemically induced stabilization of MTs leads to accelerated senescence of animal cells, a phenomenon utilized in cancer treatment. In contrast, in plants, senescence-associated cell death is accompanied by disruption of the MT network (Keech et al. 2010), and simply disrupting MTs with different pharmacological agents induces defence gene expression, possibly because MT-tethered transcription factors might be released to the nucleus (Qiao et al. 2010).

The duration of pauses between shrinkage and continued growth is one of the factors affecting cortical MT dynamics and orientation within the cortical array (Kawamura and Wasteneys 2008). The conserved eukaryotic MT (+)end proteins and MT polymerases, MAP215 and CLASP, regulate this dynamic instability (Ambrose et al. 2007, Kawamura and Wasteneys 2008). MTs and MT (+)-ends in animal and fungal cells shape the ER and endomembrane system including Golgi (Vaughan et al. 2005, Grigoriev et al. 2008), and direct the secretory COPII and endosomal traffic (Watson et al. 2005, Schuster et al. 2011). Similar MT-membrane interactions have been detected in plants (Dryková et al. 2003, Pastuglia and Bouchez 2007, Deeks et al. 2010, Hamada et al. 2012, Ambrose et al. 2013). Internalization of PM receptors with endosomes is involved in PTI signalling (Robatzek 2007). Endosomes are also involved in polarized growth of the cells via auxin signalling (Voigt et al. 2005). *Arabidopsis* CLASP interacts with a conserved endosomal recycling component and thereby mediates MT-endosome interactions (Ambrose et al. 2013). The signalling molecules belonging to the Rho family of small GTPases function in endosomal vesicle recycling (reviewed in Yalovsky et al. 2008), but also localize in ERES and participate in their assembly (Zhang et al. 2010), and control cortical MT-array organization (Fu et al. 2009). These interactions suggest a central role of MTs and MT dynamics in the cell wall – PM – endomembrane continuum and its signalling events (Fig. 2). At least one R protein of *Arabidopsis*, ZAR1, may monitor the integrity of cortical MTs because it recognizes the active HopZ1a effector, but not a mutated effector unable to disrupt MTs (Lewis et al. 2010, Lee et al. 2012). Interestingly, ZAR1-mediated HR does not depend on the previously described downstream signalling components of other R proteins (Lewis et al. 2010).

1.4.4 MT associations of plant virus movement proteins

All the well characterized MT-associated proteins of plant viruses are movement proteins. The classic example of movement proteins, MP of TMV, colocalizes with MTs (Heinlein et al. 1995), and directly interacts with tubulin and stabilizes cortical MTs (Ashby et al. 2006, Ferralli et al. 2006). In addition it interacts or colocalizes with MT end binding protein 1 (EB1)

(Brandner et al. 2008), γ-tubulin (Sambade et al. 2008), and an MT-associated and stabilizing protein MPB2C that increases MPs affinity on MTs (Kragler et al. 2003, Curin et al. 2007). MT-interactions in early and late infection may be involved in different functions in the TMV infection cycle (Niehl et al. 2013). In addition to its interactions with MTs, MP also interacts with viral RNA, ER and actin (reviewed in Niehl et al. 2013) and may also be targeted to chloroplasts, since the MP of TMV flavum strain is copurified with components of PSII from chlorotic tobacco leaves (Lehto et al. 2003).

Tobamoviral MP is expressed from a subgenomic RNA, and its accumulation kinetics differ from those of the replicase proteins expressed from genomic RNA and of CP expressed from a second subgenomic RNA (Dawson and Lehto 1990). At high temperatures, faster enlargement of infection foci, increased accumulation of MP on MTs in early infection, and lower total accumulation of MP were correlated (Boyko et al. 2000). The lower total accumulation of MP could have been due to faster initiation of movement that may have shortened the particular phase when the MP is translated (Boyko et al. 2000). Gillespie et al. (2002) studied MP mutants with different effects on TMV movement and found that improved cell-to-cell movement was correlated with a higher relative accumulation of MP within ER than at MTs.

Viral interactions with MT-associated proteins may have different roles than direct interactions with MTs. The first reports suggesting that plant viruses might require host proteins that regulate MT dynamics and organization are from the tobamoviruses TMV and *Oilseed rape mosaic virus* (ORMV) (Ashby et al. 2006, Ruggenthaler et al. 2009, Ouko et al. 2010). A tobacco line with a 15% reduction in MT growth slowed TMV movement by 25%, indicating that normal dynamics of the MT cytoskeleton are important for viral cell-to-cell movement (Ouko et al. 2010). The amounts of TMV genomic RNA were similar between the wild type and mutant tobacco plants and so no difference in virus replication was found. Overexpression of an *Arabidopsis* homolog of tobacco MPB2C, an MT-stabilizing protein, reduced the infectivity of ORMV in *Arabidopsis* (Ruggenthaler et al. 2009). Hence, the MTs play significant roles in the cell-to-cell movement of tobamoviruses, and consequently in their virulence.

The MT-associated MP2BC protein also affected the development of stomata in leaves of an *Arabidopsis* overexpression line (Ruggenthaler et al. 2009). Developmental regulation involves RNA and protein transport processes dependent on the size exclusion limit of plasmodesmata (Carlsbecker et al. 2010, Vatén et al. 2011). MP2BC of *Arabidopsis* and tobacco interact with certain transmittable homeobox transcription factors, and MP2BC of *N. benthamiana* Domin interacts with three movement-associated proteins of PVX, and with a stem-loop structure of the genomic RNA of PVX. These interactions implicate MP2BC in negative regulation of transport of non-cell-autonomous signals via plasmodesmata (Winter et al. 2007, Cho et al. 2012). Overexpression of MP2BC reduced PVX movement

and silencing enhanced it, while viral replication was not significantly affected by these alterations in protoplasts (Cho et al. 2012).

There are few reported *in planta* MT localizations by other plant viruses. They involve one translation- and two transmission-related proteins of CaMV, and one of the movement proteins of either *Sonchus yellow net virus* (genus *Rhabdovirus*) or *Potato mop-top virus* (genus *Pomovirus*) (Harries et al. 2009, Martinière et al. 2009, Min et al. 2010, Wright et al. 2010). A movement protein of *Potato leaf roll virus* (genus *Polerovirus*) is degraded in systemically infected leaves, in a proteasome- and MT-dependent manner (Vogel et al. 2007).

In conclusion, even though the early viral replication vesicles and late viral factories are detected at cortical MTs (reviewed in Niehl et al. 2013), no MT-related changes in viral replication or accumulation have been reported, except for those linked to viral movement (e.g., Cho et al. 2012). The movement proteins of plant viruses associate with MTs far more seldom than with actin or ER (reviewed in Harries et al. 2010). The distribution of viral proteins or RNA between MT sites and ER is likely to be regulated by MT- or ER-associated proteins (Cho et al. 2012, Niehl et al. 2012). The balancing of movement and replication functions of different viruses may differ.

1.5 Interactions of and resistance to Potato virus A (PVA)

1.5.1 Pathotypes of PVA

Potato virus A (PVA) is phylogenetically related to *Tobacco vein mottling virus* (TVMV) and TEV, and more distantly related to the *Potyvirus* type species PVY and its clade (Adams et al. 2005, Spetz et al. 2003). The host range diversities of the *Potyviruses* vary. PVA is restricted to a few species in the Solanaceae family, which may indicate selective constraints imposed by adaptation to a single host (potato) in the past. Systemic infection by PVA in susceptible potato induces mild symptoms. Adaptation to a single host often results in a fitness cost in other species (Elena et al. 2011). Some potyviruses, including PVY, TEV, *Plum pox virus* (PPV) and TuMV, have wider host ranges and infect several species within the Solanaceae or sometimes also in other families (Shukla et al. 1994).

Both dominant (extreme resistance or HR) and recessive resistance sources effective against PVA are known in potato and its relatives (Valkonen 1997, Hämäläinen et al. 2000). Resistance reactions in graft-inoculated commercial potato varieties including cv. King Edward, serology, and phylogenetic analyses suggest that the PVA strains can be divided into five groups, three on potato, one on tamarillo (*Solanum betaceum* Cav.) and one adapted to tobacco (Valkonen et al. 1995, Rajamäki et al. 1998, Oruetxebarria et al. 2000). A single strain naturally infects tamarillo, but the other

characterized strains are originally isolated from potato, and the origins of some strains are unknown.

Dynamics of adaptation of PVA to *Nicotiana* hosts have not been studied, but PVA strains that have been propagated via long serial passages in *Nicotiana* have a maximum of 5% of the nucleotides or amino acids changed along their genomes (Kekarainen et al. 1999) and accumulate to higher levels in tobacco than the potato isolates (Andrejeva et al. 1999). While being maintained in *Nicotiana*, these strains have lost aphid transmissibility and the ability to infect potato systemically (Valkonen et al. 1995, Rajamäki et al. 1998, Andrejeva et al. 1999). The adaptation of PVA to tobacco might be similar to that observed when a tobacco strain of TEV became adapted to pepper: dampened defence responses and increased viral fitness in the new host, and a decrease of fitness in the old host (Agudelo-Romero et al. 2008).

A collection of 21 PVA isolates readily infected tobacco (cv. Samsun) and induced mild transient symptoms in systemically infected leaves (Rajamäki et al. 1998), indicating that tobacco is relatively tolerant to PVA. A potato strain PVA-M that had lost its ability to move systemically in *Nicandra physalodes* (L.) Gaertn. (Rajamäki and Valkonen 1999, 2002) readily infects tobacco, causing typical mild mottle symptoms (Valkonen et al. 1995). Hence, the symptoms caused by PVA in tobacco are mild, independent of the origin of the isolate and whether its adaptation history is in potato or tobacco. This indicates either that any R proteins in tobacco fail to recognize the variable sites between the PVA strains, or that their signalling is suppressed by conserved functions of PVA.

1.5.2 Host protein interactions of PVA

Several interactions of potyviral proteins have been detected in heterologous systems, but few have been characterized in terms of interaction domains, localization during viral infection and infection phenotype. One well characterized interaction is that of VPg with eIF4E or eIF(iso)4E within replication vesicles, but its function is still not well understood. Nevertheless, the locations and mechanisms required for formation of potyviral replication vesicles have been characterized (Wei and Wang 2008, Cotton et al. 2009, Wei et al. 2010).

Direct interactions with host proteins have been previously described by only three proteins of PVA: HCpro, VPg and CP. HCpro-interacting protein 1 and 2 (HIP1 and HIP2) of potato interact with PVA HCpro *in vitro* and in yeast two-hybrid system (YTHS) (Guo et al. 2003), but interactions of HCpro and HIP1 or HIP2 have not been studied in plant cells.

PVA VPg interacts with fibrillarin, a necessary host factor for accumulation of PVA, in subnuclear compartments (Rajamäki and Valkonen 2009). Fibrillarin may be required for the silencing-suppression function of VPg (Rajamäki and Valkonen 2009).

HSP70 and its cochaperone CPIP (HSP40) interact with CP of PVA, as shown by co-immunoprecipitation from *N. benthamiana* leaves (Hafrén et al. 2010). CP of PVY interacts with CPIP of tobacco (Hofius et al. 2007). CPIP is a host factor required for virus multiplication, as CPIP mutant unable to interact with HSP70 interfered with PVY accumulation, and with the sustainment of PVA replication and translation (Hofius et al. 2007, Hafrén et al. 2010). Its subcellular localizations and interactions with the PVA replication complexes or virus factories have not been studied.

Little experimental information is available on the structures of potyviral proteins. Modelled 3D structures of P3 and HCpro and an experimental structure of internally disordered VPg have been presented. These have been used to elucidate the residues important for *Rsv1*-mediated recognition of P3 of SMV (Chowda-Reddy et al. 2011), the structural differences of HCpro proteins of PVY strains triggering or not triggering the Ry_{adg} resistance protein in potato (Tian and Valkonen 2013), the basis of direct interaction of VPg with eIF4E (Kang et al. 2005), and the pore-forming oligomeric structures of VPg (Rantalainen et al. 2009).

1.5.3 HIP2 is a candidate host factor for viral MT-related functions

The potato *HIP2* is homologous to two genes of *A. thaliana*, *SPIRAL2* (*SPR2*) and *SPIRAL2-LIKE* (*SP2L*), encoding MT (+)-end associated proteins that direct elongation of cells and are expressed constitutively at low levels in the whole plant (Buschmann et al. 2004, Shoji et al. 2004, Yao et al. 2008). Knockout phenotypes of *SPR2*, *spr2* and an allelic *tortifolia1*, and a knockout of *SP2L* (*sp2l*), are characterized by conspicuous spiral twisting of petioles and petals and slanting of roots (Buschmann et al. 2004, Shoji et al. 2004). The phenotype of *spr2* can be complemented by overexpression of SP2L protein (Yao et al. 2008), indicating that the genes control the same functions. SPR2 associates with the MT (+)-ends, and is required for dynamics of cortical MT (Yao et al. 2008). An antibody specific to *Arabidopsis* SPR2 recognizes a putative HIP2 from an MT-associated protein fraction purified from tobacco (Buschmann et al. 2004). SPR2 and SP2L enhance the dynamic instability of MT (+)-ends by acting as antipause factors (Yao et al. 2008).

Insights into endogenous interactions by HIP2 and SPR2 proteins are provided in studies that have made interaction screens in cDNA libraries from three plant species. A large interactome study of the plant immune system network in *Arabidopsis* (Mukhtar et al. 2011) reported that SPR2 interacts with 13 RLKs and two transcription factors (URL, signal.salk.edu/interactome/PPIN1). RLKs are membrane proteins functioning as sensors in various responses to developmental or external cues (reviewed in Afzal et al. 2008; De Smet et al. 2009) and are internalized for signalling by endocytosis (Robatzek, 2007).

Two of the SPR2-interacting RLKs, AtRKL1 and AtRLK902, are closely related but differ in their expression patterns: AtRKL1 is expressed predominantly in guard cells and hydathodes and AtRLK902 in elongation and abscission zones, associating them with defence and development, respectively (Tarutani et al. 2004a). Their closest homolog in tomato, TARK1, is a specific target of the XopN effector of *Xanthomonas campestris* pv. *vesicatoria* (ex Doidge) Vauterin *et al.*, a large alpha solenoid protein (Kim et al. 2009). The interaction between the effector XopN and TARK1 inhibits PTI (Kim et al. 2009).

Another screen for components of the flowering regulatory network indicated that tomato HIP2 might be an interaction partner of the tomato Constans-like transcription factor TCOL1 (Ben-Naim et al. 2007). This interaction, however, was tested only in YTHS and was detected with a truncated but not by a full-length TCOL1 (Ben-Naim et al. 2007).

The HIP2 homolog of sugar beet (*Beta vulgaris* L.) interacts with pathogenicity protein p25 of *Beet necrotic yellow vein virus* (BNYVV, genus *Furovirus*) (Thiel and Varrelmann 2009). This interaction has been shown to occur also *in planta* but its subcellular localization and its role in the viral infection cycle are unknown. P25 interferes with proteasome function (Thiel et al. 2012) and is responsible for the virulence of BNYVV, inducing leaf symptoms and the root proliferation symptom called rhizomania (Chiba et al. 2008, Peltier et al. 2011). Possibly, if the sugar beet HIP2 interacts with a sugar beet homolog of the root elongation-related AtRLK902, the interaction of the viral p25 with HIP2 may target this RLK and thus alter root growth in sugar beet.

The interactions of SPR2 and HIP2 proteins with the RLKs and transcription factors could indicate them as integrators or scaffold proteins of signalling networks in plant development and sensing of stress.

1.5.4 HCpro has multiple functions and a conserved structure

HCpro is required for genome amplification and movement of potyviruses (Rajamäki et al. 2004) and is able to enlarge plasmodesmata (Rojas et al. 1997). The C-terminal domain of HCpro contains the autocatalytic proteinase activity required to release HCpro from the polyprotein (Carrington et al. 1989). HCpro is a strong RNA silencing suppressor (Anandalakshmi et al. 1998, Brigneti et al. 1998, Kasschau and Carrington 1998) and interferes with the silencing pathway either by binding to siRNA molecules or inhibiting their methylation, either directly or by interacting with methylase Hua-enhancer 1 (HEN1) (Ebhardt et al. 2005, Yu et al. 2006, Lózsa et al. 2008, Jamous et al. 2011). HCpro may protect RNA also by inhibiting proteasome activity (Ballut et al. 2005, Sahana et al. 2012).

Interactions between viral proteins may indicate that they are in the same complexes in addition to their being transiently part of the viral polyprotein. RNA silencing is suppressed not only by HCpro but also by VPg (Rajamäki

and Valkonen 2009). VPg of LMV interacts with the viral helicase CI and with HCpro (Roudet-Tavert et al. 2007, Tavert-Roudet et al. 2012). VPg of LMV, PVA, *Pea seed-borne mosaic virus* (PSbMV) and ClYVV interact with HCpro of the same viruses in YTHS or *in vitro* (Guo et al. 2001, Yambao et al. 2003, Roudet-Tavert et al. 2007). CI of PVA interacts with HCpro but not with VPg in YTHS (Guo et al. 2001). HCpro also interacts with CP and acts as a bridge molecule between virions and aphid mouthparts (Blanc et al. 1998, Peng et al. 1998). These interactions suggest that HCpro collaborates with VPg, CI or CP in processes like replication, movement, encapsidation of the virion, and suppression of RNA silencing. However, the localizations and interactions of HCpro during infection are not known.

Its multiple functions in the infection cycle implicate HCpro as an important and probable target for the plant defence system. HCpro of several potyviruses is the determinant, or one of the determinants, of necrosis in infected plants. HCpro and its structure have been under strong selection, as mutations or insertions along HCpro reduce its silencing-suppression capacity and reduce the fitness or systemic movement of virus (Kasschau and Carrington 2001, Torres-Barceló et al. 2008). Hence, any interaction partners of HCpro are of interest as they might be potential candidates for durable resistance.

2 AIMS OF THE STUDY

Few host protein interactions of potyviruses have been characterized in detail. Interactions of the potyviral HCpro with few proteins have been shown in plant cells but the subcellular localizations and functions of these interactions during virus infection are poorly known.

The interaction of HCpro of PVA with the putative MT-associated protein HIP2 of potato has been shown in heterologous systems but not in plants. Furthermore, some MT-related interactions have been studied in other plant virus families, but not in potyviruses.

The interactions of cellular translation initiation factors with three potyviral proteins other than HCpro have been reported, and at the moment the incompatible alleles or forms of eIF4E genes are the most important resistance sources in plant breeding. On the other hand, MTs are involved in defence signalling. Thus, MT-associated HIP2, and translation initiation factor eIF4E proteins are promising candidates in the quest for virus resistance in plants.

The aims of this study were

- to test whether two host proteins, HIP2 and eIF4E, interact with HCpro in infected plant cells and where these interactions are located;
- to map and experimentally verify, using mutagenesis, the domains or residues of multifunctional HCpro involved in these host interactions;
- to predict the 3D conformation of HCpro of PVA and to study if mutations in HCpro alter the conformation of HCpro and whether such alterations may have pleiotropic effects on the HCpro functions in host cells;
- to test whether HIP2 is similar to the homologous MT-associated protein of *Arabidopsis*, and to predict and test functional domains of HIP2 to understand its cellular functions; and
- to determine whether the interaction of HCpro with HIP2 is important for the pathogenicity and virulence of PVA.

3 MATERIALS AND METHODS

The materials and methods used in this thesis are described in detail in publications I-IV, as indicated in Table 1.

Table 1. *The methods applied in this thesis*

Method	Publication
Design and preparation of DNA constructs	
Traditional cloning with restriction digestion and ligation	I, II, III, IV
Recombination-based cloning	I
Targeted mutagenesis and design of primers for mutagenesis	II
Cloning from cDNA	I
Construction of vectors for *Agrobacterium*-mediated transformation and protein expression in plants	I, II
Pathogenicity and virulence experiments in plants	
Virus incoulation by particle bombardment, sap-inoculation, or agroinoculation	I, II, IV
Virus detection and quantification	I, II, IV
Statistical analysis with pairwise and multiple comparisons	I, II
Virus-induced gene silencing (VIGS) using *Tobacco rattle virus*	I
Detection of reactive oxygen species by histochemical staining	II
Analysis of gene expression	
Extraction of total RNA from plants	I, II, III
Microarray and quantitative real-time PCR	I, II
Command line applications including NCBI Blast+	II
Transformation and transient expression	
Agrobacterium-mediated transformation of *Arabidopsis thaliana*	I
Selection of homozygous transgenic lines by PCR-based genotyping	I
Agroinfiltration for transient protein expression in leaves of *N. benthamiana*	I, II, III, IV
Visualization of RNA silencing suppression in leaves of *N. benthamiana*	III
Northern blot and siRNA blot	III
Analysis of protein-protein interactions and localization *in vivo*	
Yeast two-hybrid system	I, II, IV
Bimolecular fluorescence complementation	I, IV
Extraction of proteins from plant and yeast	I, II, III
Western blot for protein detection	I, II, III
Epifluorescence microscopy and confocal laser scanning microscopy	I, II, IV
Colocalization analysis using deconvolution and 3D-reconstruction	I
***In silico* analysis of protein sequences and protein structures**	
Multiple alignment of protein sequences	I, II
Phylogenetic analysis	I
Secondary and 3D structure analysis of proteins	I, II

4 RESULTS AND DISCUSSION

4.1 Domain structures of HIP2 and HCpro proteins

4.1.1 Secondary and tertiary structure prediction

This study started with the aim of characterizing the host protein interactions of PVA HCpro in detail, taking into account the multiple functions and the reported host interactions known to be mediated by potyviral HCpro. Elucidation of the normal functions of the host protein HIP2 in the plant cell was relevant to hypothesize its putative role during virus infection. These aims were expected to benefit from structural information of HCpro and HIP2. Conformational information on the secondary structures of a protein, like the order of its local α helix or β sheet elements, or the tertiary (3D) structure showing the organization of those elements, may provide clues about the protein's locations, interactions and functions and assist in the distinction of its functional or structural domains. If experimentally determined structures are not available, structural elements and 3D models of poorly known proteins can be predicted *in silico*.

Methods used for prediction of protein tertiary structures (3D) fall into three types: *ab initio* methods that calculate the best theoretical fold from the primary sequence, comparative ones based on homology to other proteins with known structure (homology modelling), and threading (fold recognition) that assesses the compatibilities of a sequence to experimentally determined structures available in a structural database, independent of homology. The performances of various prediction methods are compared biannually in Critical Assessment of Structure Prediction (CASP) experiments (URL, http://www.predictioncenter.org). The I-Tasser server has had the best accuracy (similarity between predicted and experimentally solved structure) in server benchmarking tests during several CASP experiments, including the latest conducted during 2012. The I-Tasser server (Roy et al. 2010) executes a composite method, wherein prediction of secondary structure is used as a guide in a threading step where similarities to known fold elements are sought. Based on those the target sequence is split into fragments. Short segments lacking suitable structural templates cannot be solved with threading methods, so they are predicted using *ab initio* modelling. A full-length model is assembled from the obtained structural fragments and an iterative energy refinement step is done in order to adjust them into a final model without steric clashes and of lowest energy state.

The predictions of secondary structures in the HIP2 (**I**) and HCpro (**II**) proteins suggested that they are mainly α-helical proteins with short stretches of putative β-folds. Algorithmic methods (NPS@, Combet et al.

2000), that recognize local structural elements based on experimentally determined probabilities of certain amino acids (and their neighbours) in similar structures, and artificial neural networks (Jpred; Cole et al. 2008), that usually employ a multiple alignment and are trained to detect weaker similarities at the level of sequence profiles, gave quite similar results.

A 3D model of PVA HCpro conformation was predicted using I-Tasser server (Roy et al. 2010), and had a good accuracy score (**II**). As suggested by the earlier secondary structure predictions, the fold of HCpro was found α-helical. An experimentally determined high-resolution 3D-structure of the C-terminal proteinase domain of TuMV HCpro (Guo et al. 2011) was amongst the templates. The model was later applied to explain the results obtained with mutated HCpro proteins in biological experiments (**II**). Hence, even in the absence of a suitable full-length structural template for HCpro, its likely 3D shape could be predicted. Similar 3D models of HCpro have been used to compare the conformations of HCpro proteins from different strains and mutants of PVY (Tian and Valkonen 2013).

HIP2, however, appeared to be a more difficult target, possibly because of the lack of structural templates to some of its domains, or difficulties in combining an overall conformation of a relatively large and possibly flexible α-helical protein. The score of a similarly predicted 3D model of HIP2 was hardly significant (*unpublished results*), but it had similarities to structures of large α-helical proteins, including those containing HEAT-repeats, such as importins and the PP2A subunit A. Thus, additional algorithmic and neural network–based methods were applied in order to predicts its secondary structures and domains (**I**).

4.1.2 Putative TOG and CC domains of HIP2 reveal similarities to MT end-associated proteins (I)

HIP2 and *Arabidopsis* SPR2 and SP2L belong to a protein family specific to plants (Shoji et al. 2004). To study the structure of potato HIP2 (StHIP2), its amino acid sequence was aligned with the homologous protein of tobacco, NtHIP2 (sequenced in **I**), and with *Arabidopsis* SPR2 and SP2L. Particular physicochemical regions and motif signatures relevant for MT-associated proteins were sought for, including positively charged regions, HEAT-repeats and α-helical CC motifs.

Three domains of HIP2 were distinguished at the secondary structure level. Mainly α-helical regions were detected at both the amino (NH_2)- and C-terminal domains of the protein, while a central domain contained two consecutive α-helices flanked by unstructured regions (**Fig. 3A**). When tested with an algorithmic method (Lupas et al. 1991), the helices in the central region were predicted to form a CC motif. In order to investigate any domainwise variation in physicochemical properties (charge and isoelectric point), protein charge blots were drawn by counting local mean charges of the residues along the polypeptide (Drevensek et al. 2012). The C-terminal

domains of HIP2, SPR2 and SP2L were predominantly acidic or negatively charged, while their NH_2-terminal ends and parts of the disordered regions were basic or positively charged (**Fig. 3B**).

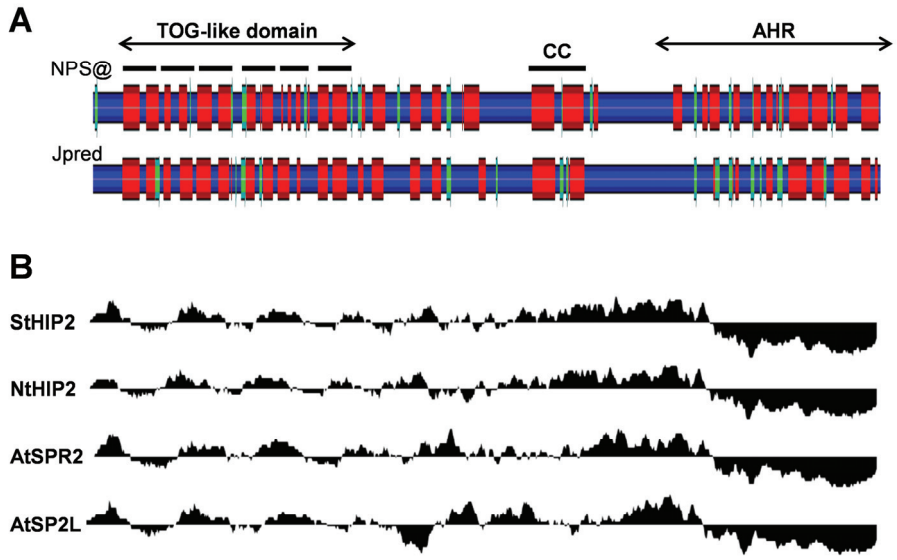

Figure 3 Structural domains and elements of HIP2, SPR2 and SP2L. **A.** Comparison of secondary structure predictions on HIP2 made with NPS@ or Jpred methods. The red bars indicate the probability of α helix and the green bars the probability of β sheet elements. Putative structural domains are marked with horizontal arrows: TOG, tumour overexpressed gene domain, CC, coiled-coil, AHR, α-helix rich domain. Positions of HEAT repeats and the CC are marked with horizontal lines. **B.** Protein charge blots demonstrate that StHIP2, NtHIP2, SPR2 and SP2L have similar negatively charged C-terminal domains and positively charged regions of various lengths in the other parts of the protein. Values below or above the baseline indicate negative or positive average charge, respectively.

More structural information was further obtained from the NH_2-proximal α-helical region. The SPR2 family has been predicted to contain HEAT repeats. Buschmann et al. (2004) suggested five HEAT repeats and a CC motif in each of the proteins of SPR2 family, while Shoji et al. (2004) predicted nine HEAT repeats in SPR2 or SP2L. To analyse HEAT or related ARM repeats in HIP2, candidate fragments were defined, extracted from the alignment of StHIP2, NtHIP2, SPR2 and SP2L and validated based on similarity to repeat profiles using neural network tools and a training set as described by Kippert and Gerloff (2009). Of 13 HEAT or HEAT-like candidate fragments found during an initial screening (*not shown*), six in the NH_2-proximal alpha-helical region met the validation criteria of Kippert and Gerloff (2009) (**Fig. 3A**; and Supplementary Fig. 1 in **I**). Because HEAT-repeats often occur as multi-repeat arrays and interact with the neighbouring repeats, the packed array is energetically favourable. Consequently, the

individual HEAT repeats can exhibit high sequence variability and are difficult to detect from the sequence (Andrade et al. 2001, Knutson 2010). Thus, the six repeats were predicted with confidence, but the presence of additional HEAT repeats remains possible.

When the NH_2-proximal region of HIP2 containing the HEAT repeats was subjected to profile-profile comparisons using HHpred search tool (Söding et al. 2005), significant structural similarities were found to sequence profiles and experimentally solved 3D structures of TOG or TOG-like domains in protein family (PfamA) and protein structure (PDB) databases, respectively (**I**). A TOG domain is formed of six or seven consecutive HEAT-repeats (Al-Bassam et al. 2007, Ayaz et al. 2012). Two related TOG-domain protein families, MAP215 and CLASP, are conserved across eukaryotic kingdoms. The members of the MAP215 family are tubulin polymerases and promote MT assembly, while the members of CLASP family inhibit catastrophic depolymerisation and are MT rescue factors (Andrade et al. 2001, Slep and Vale 2007, Al-Bassam and Chang 2011). TOG-domains in these MT (+)-end associated proteins are required for interaction with unincorporated (soluble) tubulin heterodimers and are essential for functions of MAP215 and CLASP in MT dynamics. A predicted TOG-domain in HIP2 and SPR2 is consistent with the observed action of SPR2 in promoting MT polymerization at MT (+)-ends (Yao et al. 2008).

The means by which various MT-associated proteins track the growing MT (+)-ends is by interacting with the conserved MT (+)-end protein EB1. *Arabidopsis* EB1 concentrates at growing and shrinking MT (+)-ends and at nucleation sites marking the slower MT (-)-ends (Chan et al. 2003). EB1 directly recognizes the tubulin conformation of MT (+)-ends and acts as a general adaptor in the MT-associated protein interaction network that is critical for dynamics and cellular interactions of MTs (Kumar and Wittmann 2012). A sequence corresponding to the EB1 interaction motif (S/T)x(I/L)P (Kumar and Wittmann 2012) was discovered in the fifth HEAT repeat of the TOG-domain of StHIP2 (SLLP, aa 328-330, *unpublished results*). The motif was conserved in HIP2, SPR2 and SP2L but not in other members of the SPR2 family. The EB1-binding motif is also found in CLASP proteins and in a MAP215-interacting protein, and although it is not required, it may enhance the MT (+)-end localization of CLASP and MAP215 (Kumar and Wittmann 2012).

In conclusion, two types of α-helical repeat motifs, HEAT and CC, were found in HIP2 and the predictions suggested that HIP2 could contain three structural domains. The predictions indicated analogy to other MT-associated proteins with similar functions in MT dynamics and suggested that HIP2 may engage in both direct and indirect interactions with the EB1-associated protein group that accumulate at MT ends and are relevant for cellular functions of MTs. The previously known TOG-domains are limited to the MAP215 and CLASP families conserved in eukaryotes; hence the discovery of a putative TOG domain in HIP2, a member of a plant-specific

family, is of particular interest. The results of structural and domain predictions made *in silico* were critical for interpreting the results obtained using *in vivo* methods, as will be shown below.

4.1.3 A structural model and functional domains of HCpro (II)

A high level of conservation within central and C-proximal regions was evident when amino acid sequences of HCpro proteins from 47 potyviruses were aligned (Supplementary Fig. 2 in **II**). Interestingly, a conserved hydrophobic region (*unpublished results*) overlapped an eIF4E binding motif (determined in **IV**). This hydrophobic region may signify a conserved interaction pocket, or it could be involved in membrane interactions, a common property of viral movement proteins (Harries et al. 2010),. Membrane interactions of HCpro are also indicated by structural and organizational changes of ER induced by expression of TuMV HCpro (Zheng et al. 2011). The hydrophobic region could also be related to the close association of HCpro with 6K2-vesicles (**IV**).

Structural studies indicate that HCpro folds to three structural domains and may oligomerize via alternate domain-to-domain interactions (Plisson et al. 2003, Ruiz-Ferrer et al. 2005). An NH_2-proximal region (approx. 100 amino acids) forms a structurally independent domain required for aphid transmission (Dolja et al. 1993) and for interactions with a subunit of a 20S-proteasome (Jin et al. 2007a). Central and C-terminal domains (approx. 200 and 250 aa, respectively) are mainly α-helical (Plisson et al. 2003). The central domain harbours conserved motifs required for interactions with CP and RNA, the C-terminal domain has autoproteolytic activity, and these two domains may interact in the 3D conformation of the protein (Peng et al. 1998, Urcuqui-Inchima et al. 2000, Ballut et al. 2005, Shiboleth et al. 2007, Jamous et al. 2011). Mutations within the central region of HCpro affect virus amplification and systemic movement. The reduced accumulation of these mutants is in most cases correlated with ineffective binding to siRNA or otherwise reduced RNA silencing suppression capacity of the mutated protein (Kasschau and Carrington 2001, Torres-Barceló et al. 2010a, 2010b).

The predicted structural elements in HCpro varied slightly between different prediction methods (**Fig. 4**). The greatest number of differences was observed within a flexible hinge region that is located within the central domain and connects the central and C-terminal domains (Plisson et al. 2003). While the secondary structure predictions indicated several β-sheet elements, the HCpro tertiary structure created with I-Tasser contained only short β-turns (not depicted in Fig. 3 or Fig. 4). This may reflect a bias or lack of suitable templates available in the protein database, or suggest that at least one of the HCpro conformations is mainly alpha-helical. Even identical amino acid sequences may fold differently, and especially flexible regions may be able to adapt to several alternative conformations *in vivo* (Branden and Tooze 1999). Some β-sheet elements in HCpro have been proposed

based on structural similarities with an RNP-like RNA-binding domain (hinge domain) and with a papain-type protease domain (C-terminal domain), respectively (Urcuqui-Inchima et al. 2000, Guo et al. 2011). For example, two short β-sheet elements in the crystallized protease active site of TuMV HCpro indicate resemblance to a papain-type proteinase fold (Guo et al. 2011) but those β-sheets did not appear to exist in the PVA HCpro model created in this study. However, the catalytic histidine residue (His417) in the proteolytic active site that is within a β-sheet in the experimental structure of the TuMV HCpro, located within a β-turn in the PVA HCpro model.

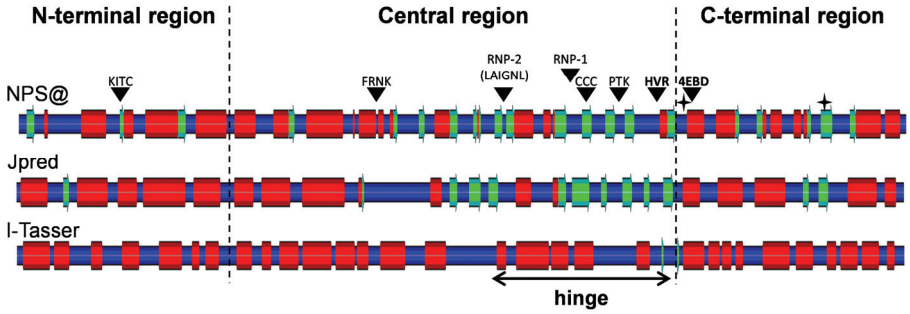

Figure 4 Comparison of secondary structure elements predicted in PVA HCpro using NPS@, Jpred and those of the 3D fold model made with I-Tasser. Red bars indicate probable α-helical and green bars β-sheet elements. β turn structures are not shown. Particularly the hinge region within the central domain contains several conserved functional motifs (arrowheads). Positions of the two conserved catalytic residues of the C-terminal protease domain are marked with stars. Highly variable region (HVR) and eIF4E-binding motif (4EBD) were determined in this study. RNP, RNA-binding domain.

4.2 HIP2 is functionally related to MT-associated protein SPR2 (I)

SPR2 and *SP2L*, which belong to the *SPR2* family of six genes in *A. thaliana* (Buschmann et al. 2004, Shoji et al. 2004), are recently diverged from each other and control the same functions (Yao et al. 2008). To identify which of the *SPR2* family members are most closely related to the HIP2 from Solanaceae and whether HIP2 has same function as SPR2 and SP2L, the putative orthologs were compared by reverse genetics: a phylogenetic comparison and genetic complementation test between *SPR2* and *HIP2* were done, and a silencing phenotype of *HIP2* and subcellular localization of HIP2 protein were compared to those of *SPR2* and SPR2 protein, respectively. To broaden the scope of this study, the cDNA-clone of *HIP2* from tobacco was included in the interaction and localization experiments.

4.2.1 HIP2 is a functional ortholog of SPR2 and SP2L

While the present study was in progress, genomic sequences of some of the species in the family Solanaceae became available, including the genomes of tomato and an almost complete sequence of a doubled-monoploid line of potato (URL, http://www.potatogenome.net), as well as a draft genome of *N. benthamiana* as a first representative of genus *Nicotiana*. Sequence comparisons in the SOL genomics network (URL, http://solgenomics.net) discovered four *HIP2*-homologs amongst the predicted gene models in the diploid potato and tomato (*unpublished results*). In both species, a single gene was highly similar to *Arabidopsis SPR2* and *SP2L*, while the other homologous genes were more similar to the other members of the *Arabidopsis SPR2* family. In *N. benthamiana*, an allotetraploid species, blast searches discovered 8 putative *HIP2* family members (**I**), twice the number of homologous genes in the diploids. As expected, two of these eight genes were highly similar to *SPR2*, *SP2L* and *HIP2*, being likely homeologs of *HIP2*.

A phylogenetic analysis of *StHIP2*, *NtHIP2* and the *Arabidopsis SPR2* family confirmed that *HIP2* belongs to the same branch as *SPR2* and *SP2L*. These results were consistent with *SPR2* and *SP2L* being paralogs of each other and with Solanaceae *HIP2* being an ortholog to both of them. In *Arabidopsis*, SPR2 and SP2L are redundant and the *spr2 sp2l* double mutant has a more severe twisting phenotype than either of the single mutants (Yao et al. 2008). Both genes are expressed in whole plants, but in most tissues *SPR2* mRNA is detected at higher levels than that of *SP2L*, except, e.g., in root hairs where *SP2L* has a higher expression (Yao et al. 2008). These differences suggest sub-functionalization (differential expression of duplicated genes), a more common evolutionary consequence than neo-functionalization (Roulin et al. 2013).

The two *HIP2* genes in *N. benthamiana* were targeted with the virus-induced gene silencing (VIGS) system based on *Tobacco rattle virus* (TRV, genus *Tobravirus*) (Ratcliff et al. 2001). The *HIP2*-silenced *N. benthamiana* plants developed a spiral phenotype including twisted leaf petioles (Fig. 3 in **I**) and twisted flower tubes (*unpublished results*). To initiate silencing, stretches of over 20 nt identity between a silencing inducer and target are usually required (Thomas et al. 2001), which was met with the fragment of *NtHIP2* that was used as a silencing inducer. The nucleotide identities of *NtHIP2* with the other *SPR2* or *HIP2* family members were lower (45-50%) and stretches of identity were shorter than 10 nucleotides.

Arabidopsis spr2 was transformed with a construct containing the protein-coding sequence of potato *HIP2* flanked by the promoter and 3' proximal regions of *Arabidopsis SPR2*. In the HIP2-transgenic lines, the twisting phenotype of *spr2* was rescued and plants exhibited normal architecture (Fig. 1 in **I**).

The similarity of the phenotypes of *SPR2* knockout and *HIP2* silenced plants supported the idea of functional homology of these genes. But this

evidence alone was not sufficient to draw that conclusion, because interference of several genes, particularly those related to MT functions, can have similar phenotypes (Hashimoto 2002). In classical genetics based on progeny analysis, a complementation (or *cis–trans*) test invented by Seymour Benzer and Ed Lewis (reviewed by Hawley and Gilliland 2006) can reveal whether an identical recessive phenotype in two strains is induced by defects in the same locus, and is conducted simply by crossing the strains to determine the phenotype of the progeny. When a gene sequence is known, functional similarity of homologous genes across species can be tested using a reverse genetics approach: transforming the gene of interest to a characterized knock-out line of another species. Therefore, the phenotypic complementation observed in several independent HIP2-transgenic lines together with the phylogenetic evidence confirmed that HIP2 shared a similar function as SPR2, i.e., is homologous and functionally related.

4.2.2 HIP2 localizes at cortical MTs *in planta*

Arabidopsis SPR2 and SP2L have been characterized as MT-associated proteins that directly bind tubulin *in vitro* and localize to MTs *in vivo* (Buschmann et al. 2004, Shoji et al. 2004, Yao et al. 2008). NtHIP2 and StHIP2, with fusion to monomeric red fluorescent protein (mRFP), were transiently expressed in plant leaves by agroinfiltration, that is, by means of gene transfer by *Agrobacterium tumefaciens* (Smith & Townsend, 1907) infiltrated into the leaves. The fluorescence signals of HIP2 were observed with fluorescence microscopy in filamentous and punctuate structures in the peripheries of leaf epidermal cells (Fig. 2 and Supplementary Fig. 3 in **I**) and were aligned with fluorescently labelled MTs at a cortical MT array (Fig. 2 in **I**), indicating localization of HIP2 at cortical MTs.

4.3 Self-interactions of HIP2 (I)

4.3.1 HIP2 self-interacts on MTs

HIP2 was predicted to contain a putative TOG or TOG-like domain, but all of the characterized eukaryotic TOG-domain proteins contain more than one TOG domain. Amongst the MAP215 proteins, XMAP215 of *X. laevis* functions as a monomer and contains five TOG domains. Five TOG domains are found also in Zyg9 of roundworm *Caenorhabditis elegans* (Maupas, 1900), MOR1 of *Arabidopsis* and MSPS of common fruit fly *Drosophila melanogaster* (Meigen, 1830). Stu2p of baker's yeast *Saccharomyces cerevisiae* (Meyen ex E.C. Hansen) contains only two TOG domains, but it functions as a dimer (Al-Bassam and Chang 2011). Also the CLASP family proteins have 2-3 TOG-like domains each (Al-Bassam and Chang 2011). The yeast CLASP, Clsp1, functions as a dimer, and its two TOG-like domains are

required for tubulin heterodimer binding in the same way as the TOG domains of MAP215 proteins (Al-Bassam et al. 2010).

Because of the punctuate localization pattern of fluorescently labelled *Arabidopsis* SPR2, the formation of SPR2 complexes within cortical MTs was suggested by Shoji et al. (2004), but self-interaction was not tested. In the present study, self-interactions were studied using two *in vivo* interaction tests, YTHS in yeast cells and bimolecular fluorescence complementation (BiFC) in the epidermal leaf cells of *N. benthamiana*. For BiFC, the proteins were coexpressed in fusion with N- or C-terminal halves of yellow fluorescent protein (YFP) via agroinfiltration.

StHIP2, NtHIP2 and SPR2 self-interacted in yeast (Supplementary Fig. 5 in **I**) and in leaf cells where the self-interaction of HIP2 occurred along fluorescently labelled MTs (Fig. 2 in **I**). To determine the localization at the maximal resolution of light microscopy, a series of optical sections spanning the cortical MT were acquired with confocal scanning microscopy and mathematically resolved using deconvolution algorithms. Subsequent colocalization analysis confirmed visual observation of overlapping signals: HIP2 was located along MTs and particularly tended to accumulate at MT intersections (Fig. 2 and Supplementary Movie 1 in **I**).

Similar localizations have been reported for SPR2 but also for other MT (+)-end proteins of plants, which are not limited to MT ends but occur along cortical MTs and accumulate at crosses or branches of MTs when expressed at natural levels (Nakajima et al. 2004, Kawamura et al. 2006, Kirik et al. 2007, Yao et al. 2008,). Localizations of MT (+)-end proteins of plants may be similar to those of the γ-tubulin complex that marks the positions of prospective MT nucleation sites, MT branches and the points of MT-membrane contacts (Dryková et al. 2003). In other eukaryotes, the members of MAP215 and CLASP families are observed, in addition to MT (+)-ends, also at MTOC and along MTs (reviewed by Al-Bassam and Chang 2011). This implied that the observed localizations of HIP2 represented a natural subcellular distribution of an MT (+)-end protein.

4.3.2 Self-interactions affect HIP2 localization within MT array

To better understand the cellular functions of StHIP2, the domains responsible for self-interaction and MT association were determined. The structural predictions on HIP2 were applied to design truncated forms of the StHIP2 containing the NH_2-terminal TOG-domain, the central CC domain, or a C-terminal α-helix rich (AHR) domain. Self-interaction tests using YTHS and BiFC consistently indicated that there were two distinct self-interaction domains, or conformations, in HIP2 (Fig. 5 in **I**). The truncated StHIP2 proteins containing the TOG-domain and the CC (TOG-CC) self-interacted and appeared to be localized along filaments *in planta*. The C-proximal AHR fragments did not self-interact, but interacted with the TOG-CC fragments and appeared as a more punctuate pattern (Fig. 5C in **I**). These results show

that the TOG-CC fragment alone was sufficient for MT association and localization along the MT. AHR was not required for MT localization but was required for the punctuate localization pattern that coincided with MT intersections.

Experiments conducted in YTHS using the three above-mentioned and two additional truncated HIP2 proteins suggested that the CC domain was required for self-interaction (Fig. 5B in **I**). Similarly, dimerization of Stu2p is mediated by a CC domain (Al-Bassam et al. 2006). Dimerization based on CC motifs can occur via formation of a four-helix-bundle structure, as in the dimerized RNA binding protein ROP (Branden and Tooze 1999).

TOG-domain proteins bind to free tubulin heterodimers, but upon incorporation into an MT lattice, the tubulin heterodimer experiences a major conformational change (reviewed in Buey et al. 2006). Consequently, the interaction of a TOG domain with a soluble tubulin dimer and the interaction of a whole protein with tubulin polymer (= affinity to MT-lattice) have to be structurally separate or mediated by separate domains in the MT-associated protein (Ayaz et al. 2012). MT lattice interactions often depend on basic, positively charged regions or exposed loop structures (Nakaseko et al. 1996, Culver-Hanlon et al. 2006, Currie et al. 2011, Widlund et al. 2011, Drevensek et al. 2012, Lechner et al. 2012). In HIP2, such positively charged regions were found within the TOG and CC domains but not within the AHR domain.

Yao et al. (2008) demonstrated that a truncated SPR2 protein containing an NH_2-proximal region that overlaps with the TOG domain found in the present study efficiently binds MTs *in vitro*. However, it, or two other fragments of SPR2 that contain the central or C-terminal domains failed to localize to MTs when expressed *in planta*. Thus, the results presented by Yao et al. (2008) and in the present study (**I**) could indicate that the TOG and the CC domains together are required for efficient MT-localization *in vivo* and suggest that the *in vivo* MT-localization of HIP2 or SPR2 requires self-interaction. Deletion studies on MAP215 and CLASP family proteins indicate that a minimal functional protein (a minimal MT polymerase required for rapid MT growth or MT rescue, respectively) consists of two TOG-domains and a domain or motif involved in MT-lattice binding (Al-Bassam et al. 2010, Currie et al. 2011, Widlund et al. 2011, Lechner et al. 2012). Two TOG-CC fragments of HIP2 dimerized via their CC domains could putatively represent the equivalent of such a basic functional unit (**Fig. 5**).

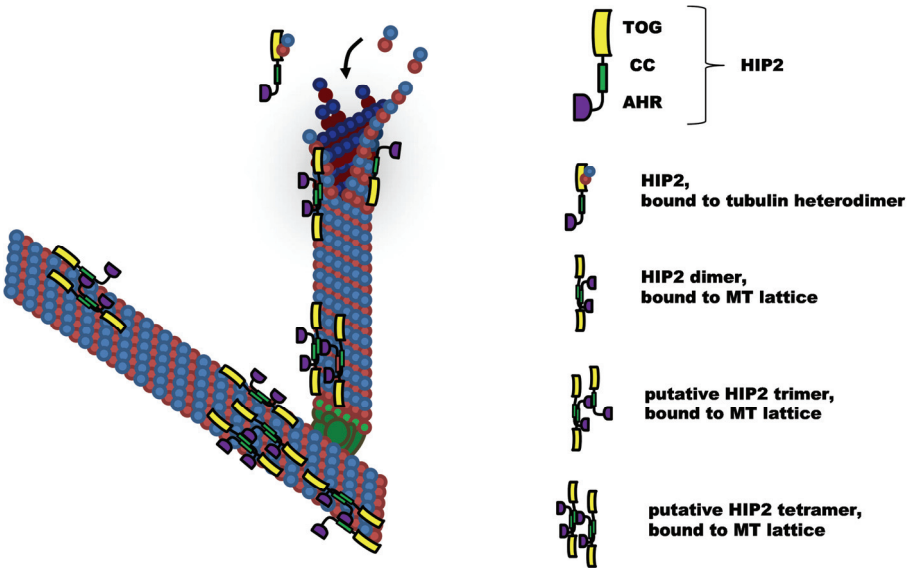

Figure 5 A model of HIP2 domain structure, self-interactions and putative associations with tubulin and MT-lattice. Structural domains: TOG, putative tumour overexpressed gene domain, CC, coiled-coil, AHR, α-helix rich domain. Black lines flanking CC indicate basic, disordered regions. The illustrated functions and distribution of HIP2 along MTs are based on eukaryotic multi-TOG-domain proteins CLASP and MAP215, in which TOG domains interact with soluble tubulin and weakly with MT lattice, basic domains interact with MT lattice, and CC domain mediates self-interactions.

Deletion studies on MAP215 proteins have shown that each of the five TOG domains of XMAP215 and MSPS, and the dimerization of the Stu2p that contains two TOG domains, contributes to the MT affinity of these proteins (Al-Bassam et al. 2006, Currie et al. 2011, Widlund et al. 2011). The MT lattice interactions and the number of the TOG domains together influence the spatial distribution of the MAP215 proteins, which appears to regulate MT growth, shrinkage and rescue events in the MT array (Currie et al. 2011, Widlund et al. 2011). Similarly, the self-interactions of HIP2, if occurring *in trans*, could be the means to bring several TOG domains together via homo-oligomerization of HIP2 (Fig. 5). The number of TOG domains and their conformation may affect the activity of HIP2 complexes on MT dynamics. AHR of HIP2 appears to be required for the uneven distribution of HIP2 on the cortical MT array. Hypothetically, one of the roles of HIP2 oligomers or complexes observed in MT intersections might be to maintain reservoirs of MT polymerization factors and to deploy them during the rapid MT array reorganization that occurs at the onset of stress signalling. The putative HIP2- or SPR2-interacting signalling or transcription-related host proteins may also be stored at those locations.

4.4 Subcellular localizations of HCpro are altered in interaction with HIP2 or eIF(iso)4E

The interactions involving HCpro and their localization in plant cells were studied using the BiFC technique. To this end, the NH_2-terminal ends of HCpro proteins were expressed in fusion with one half of YFP and coexpressed with the HCpro, HIP2 or eIF4E proteins that were in fusion with the other half of the YFP. In the case of HCpro of PVA, the tagged HCpro was expressed also from modified infectious clones of PVA (Fig. 4A in **I**), allowing observation of the HCpro interactions during PVA infection.

4.4.1 HCpro self-interacts in cytoplasm (I, II, IV)

A dimer is the likely biologically active from of HCpro in aphid transmission (Thornbury et al. 1985) as well as in other functions (Plisson et al. 2003). HCpro self-interaction was used as a positive control in both of the *in vivo* interaction methods applied in this study, YTHS and BiFC (**I**, **II**, **IV**). Self-interactions of several potyviral HCpro proteins, including HCpro of PVA, have been studied using YTHS (Guo et al. 1999, Urcuqui-Inchima et al. 1999, Kang et al. 2004, Lin et al. 2009, Zheng et al. 2011). Self-interactions of HCpro of TuMV and of fragments of HCpro of PPV have been detected also *in planta* (Zheng et al. 2011, Zilian and Maiss 2011).

The BiFC fluorescence indicating self-interaction of HCpro of PVA, TEV, PVY or PSbMV was observed in the cytoplasm and occasionally in granules (Supplementary Fig. 3 in **II**). In PVA-infected cells, the fluorescence indicating self-interaction of PVA HCpro was distributed in the cytoplasm and occasionally observed in small foci (Supplementary Fig. 3 in **I**), suggesting that HCpro may follow the ER and form ER-associated granular bodies, as described by Zheng et al. (2011) for HCpro of TuMV.

4.4.2 HCpro and HIP2 interact at cortical MTs (I)

The interaction between StHIP2 and PVA HCpro has been detected using the YTHS and *in vitro* methods (Guo et al. 2003), but not studied in plant cells. The NtHIP2 cloned in this study interacted with HCpro in YTHS (**I**), indicating that interaction of HIP2 with HCpro may be common for the two host species of PVA.

StHIP2 and NtHIP2 interacted with HCpro in living plant cells as well, as studied using BiFC. Three days post-inoculation (dpi) the fluorescence indicating interactions of the HIP2 proteins with HCpro occurred in cell peripheries and formed punctuate and filamentous patterns in both virus-free and infected cells. The fluorescence signals derived from the interaction and from a fluorescent marker for MTs overlapped (Fig. 4C in **I**) and a colocalization analysis confirmed that HIP2-HCpro interaction occurred along MTs and at MT intersections (Supplementary Fig. 4C in **I**). The HCpro

proteins of two other potyviruses, PVY and TEV, similarly interacted with StHIP2 and NtHIP2 in cell peripheries, whereas HCpro of PSbMV that poorly infects solanaceous species did not (Supplementary Fig. 3 in **II**).

As explained in the section 4.4.1, the self-interacting HCpro was localized in the cytoplasm without discernible concentration of HCpro proteins on MT-like filamentous structures. Nevertheless, HCpro accumulates to a high level while endogenous HIP2 is present at very low amounts (Buschmann et al. 2004). Overexpression of StHIP2 or NtHIP2, tagged with mRFP, markedly altered the distribution of signals from self-interacting HCpro in the PVA-infected cells. The signals from HCpro self-interaction and from HIP2 overlapped in cortical cytoplasm in a punctuate and filamentous manner (Supplementary Fig. 3 in **I**). This experiment confirmed that HIP2 may recruit at least a proportion of HCpro to cortical MTs. While interactions occurring in the BiFC system may be irreversible once the interaction between the tested proteins has allowed reconstitution of the fluorescent protein (Kerppola 2006), the results of this experiment confirmed that the localization of HIP2-HCpro interactions on MT were not an artefact of the BiFC system.

Guo et al. (2003) showed that a truncated StHIP2 protein containing CC and AHR domains interacts with HCpro in yeast cells. In the present study, the AHR domain was shown to be required and sufficient to mediate the HCpro interaction both in yeast and in plant cells (Fig. 5 in **I**). While the signals indicating interactions of full-length StHIP2 with HCpro were detected in cell peripheries along filaments and in punctuate bodies, interaction with the C-proximal AHR fragment appeared evenly distributed in the cytoplasm (Fig. 5D in **I**). This observation was consistent with the acidic nature of the AHR domain being unlikely to associate with MTs on its own. Hence, in addition to regulation of HIP2 distribution within the MT array, the AHR domain engages in a heterologous interaction.

Signals of HIP2–HCpro interactions in PVA-infected cells were observed, not only with cortical MTs, but also in larger granules near nuclei and chloroplasts at 4 dpi (Fig. 4B in **I** and *unpublished results*). The latter localizations may be related to the putative perinuclear viral factories detected at four days after agroinoculation of TuMV (Grangeon et al. 2012).

The present study was the first to report that a potyviral protein interacts with an MT-associated protein *in planta* and localizes at MTs. The TOG-CC domains of HIP2 direct HCpro to cortical MTs where HCpro accumulates at MT-MT intersections with HIP2. The occurrence of the interaction in infected cells suggested that it may have a role in the viral infection cycle.

4.4.3 Localizations of HCpro and eIF(iso)4E are altered in infection (IV)

In the present study, the eIF4E and eIF(iso)4E interactions with HCpro were initially discovered using YTHS and confirmed *in planta* using BiFC (**I**). In the non-infected cells, the HCpro-eIF4E interactions appeared evenly

distributed in the cytoplasm but observation of the interaction between HCpro and a tobacco eIF(iso)4E in the infected cells revealed that the subcellular localization was altered: the majority of interaction signals were observed in small granules and often associated with chloroplasts (Fig. 4 in **IV**), indicating that some process of the virus infection altered the localization of HCpro and eIF4E. Similar altered localizations are expected for host proteins recruited for replication or translation of potyviruses.

4.4.4 The protein complexes containing HCpro may associate with viral replication vesicles (I, IV)

During the course of the present study, subcellular localizations of 6K2 of PVA were compared with those reported for 6K2 and 6K2-associated replication vesicles of other potyviruses. Studies using TEV and TuMV have shown that 6K2 induces formation of the ER-derived vesicles that are initiated at ERES, associated with Golgi, and transported using the COPII-dependent early secretory pathway and actin-mediated transport to the vicinity of chloroplasts, induces chloroplast invaginations, and later accumulates in putative viral factories that form in association with chloroplast and endomembrane amalgamations (Schaad et al. 1997, Wei and Wang 2008, Cotton et al. 2009, Wei et al. 2010, Grangeon et al. 2012). Fluorescently labelled 6K2 of PVA followed similar pathways, as it colocalized with ER, Golgi, actin, and finally accumulated on chloroplasts and amongst agglomerated chloroplasts (**I**, **IV**, *unpublished observations*).

The signals from interacting tobacco eIF(iso)4E and HCpro formed foci close to chloroplasts, near or overlapping with signals from 6K2, indicating that the interaction associated with replication vesicles (**IV**). The eIF4E is detected in 6K2 vesicles (Beauchemin et al. 2007) and eIF(iso)4E colocalizes with the replicative dsRNA form of TuMV genome in infected cells (Cotton et al. 2009). There are no previous studies of localization of HCpro in relation to replication or translation complexes or 6K2 vesicles. Of the other potyviral proteins, NIa and NIb associate with the replication vesicles, while CI forms spike-like inclusions separate but near the replication vesicles (Wei et al. 2010). Another study suggests that the majority of CI and CP are found near but not fully colocalized with the TuMV dsRNA (Cotton et al. 2009).

Some 6K2 replication vesicles of PVA were detected in the cortical MT array near HIP2-HCpro-interaction signals (Fig. 4D,E in **I**). However, those were only a portion, indicating that their putative MT association may be partial or temporary. HIP2-HCpro interaction signals were also detected near chloroplasts that were close to the anticlinal or periclinal cell walls (Fig. 4 in **I**). As chloroplasts were observed based on their autofluorescence, alterations in their morphology were not detected.

The involvement of the MT cytoskeleton in RNA transport and processing is known in yeast and animal cells, but similar studies are lacking in plants. Many RNA-binding or translation-related proteins interact with tubulin or

MTs, as indicated by proteomic analysis of plant extracts captured in tubulin-affinity columns (Chuong et al. 2004). A putative RNA-metabolism and export factor from tobacco has been discovered using tubulin sedimentation (Hamada et al. 2009). In plants, the putative MT interactions of the RNA-associated proteins may be involved in the distribution and transport of RNA granules in the cytoplasm or in regulation of translation by capture of translation complexes or P-bodies (Hamada et al. 2009, 2012). Several RNA-related proteins whose MT-associations were discovered in the above-mentioned studies, including eIFs, poly-A binding protein, HSP70 and DEAD-box helicases, interact with the potyviral NIa, NIb, CP or CI proteins, and are recruited to 6K2 vesicles during infection (Wittmann et al. 1997, Schaad et al. 2000, Léonard et al. 2004, Beauchemin and Laliberté 2007, Beauchemin et al. 2007, Dufresne et al. 2008, Hafrén et al. 2010, Huang et al. 2010, Tavert-Roudet et al. 2012). Hence, in infected cells these proteins are taken in replication vesicles, and no MT localizations have been reported.

The COPII transport pathway, which is necessary for the 6K2 of TuMV to reach cell peripheries (Grangeon et al. 2012), is part of a secretory pathway. Disruption of the early secretory COPII pathway or the recycling COPI pathway, or inhibition of actin transport, reduces viral accumulation (Wei and Wang 2008, Wei et al. 2010). Similar effects were not observed when the MT cytoskeleton was chemically disrupted (Cotton et al. 2009). Presumably, 6K2 may arrive along actin or through the Golgi to the vicinity of the cortical MT array, as actin microfilament (MF) and MT cytoskeletons are co-ordinated. Other kinds of MT-associated vesicles have been observed in the cortical cytosol in studies using electron microscopy (e.g., Crowell et al. 2009, Gutierrez et al. 2009, Kaneda et al. 2010). For example, Golgi bodies containing cellulose synthase complexes are transported to cell peripheries along actin filaments, but upon reaching a correct site, they pause on MTs while the cellulose synthase complexes are inserted into the PM (Crowell et al. 2009). Actin localization and assembly may occur along MTs (Barton and Overall 2010, Sampathkumar et al. 2011), and interactions of actin and MTs are mediated by, among others, some kinesins and formins (Deeks et al. 2010, Frey et al. 2010, Li et al. 2010). Furthermore, the organelle positions are dictated by collaboration of actin and MT cytoskeletons, as movement and anchorage of chloroplasts require actin and MTs, respectively (Chuong et al. 2006; Takagi et al. 2009). Similarly, the movement of the nucleus is regulated via MTs (Frey et al. 2010).

Drawn together, the observed direct and indirect associations of HCpro with MTs, viral replication vesicles, actin, and with the possibly replication-associated host protein eIF(iso)4E, are in agreement with a proposed role of plant cortical MTs as platforms for cargo loading and unloading or cargo exchange (Hamada et al. 2012).

4.5 Short motifs in HCpro are involved in interactions with HIP2 and eIF4E

4.5.1 HIP2 interaction is regulated by a loop-like highly variable region (II)

Full-length and truncated HCpro proteins from two strains of PVA were used to define the HIP2-interaction area in HCpro using YTHS. Results suggested that a region of 30 amino acids near the boundary between the central and C-terminal domains of HCpro (aa 325-354) was required for HIP2-interaction (Fig. 1 in **II**). This region contained both conserved and non-conserved amino acids (Fig. 2A and Supplementary Fig. 2 in **II**). HCpro of the two strains of PVA interacted with different strengths with HIP2, and while they differed in five amino acids, only one of the differences was located within the HIP2 interaction region (Fig. 2B in **II**). This amino acid is the first residue of a highly variable region (HVR) of six non-conserved amino acids (aa 330-335).

The HVR in HCpro of PVA was mutated to resemble the HVR in HCpro of PSbMV that did not interact with HIP2. Four kinds of substitutions were made: all six residues were converted to PSbMV-like (mutant HCmABC), or only a single residue (mutant HCmA), four (HCmB), or three residues (HCmC) were substituted (Fig. 2C in **II**). The HVR mutants retained self-interactions but their HIP2-interactions were weakened: HCmABC did not interact with StHIP2 or NtHIP2, HCmB interacted weakly, while HCmA or HCmC had almost normal interactions with HIP2 proteins in YTHS. These results confirmed that the HVR regulates interaction of HCpro with HIP2.

Amino acid sequences of the HVR differed widely between the HCpro proteins interacting (PVA, PVY and TEV) and not interacting (PSbMV) with StHIP2 and NtHIP2. All six amino acids in HVR were different between PVA, TEV and PSbMV. The first residue (lysine, K) was identical in HVR of PVY and PSbMV, and the fourth residue (serine, S) was identical in HVR of PVY and PVA. Even the locations of the negatively charged residues alternated between the second and fifth positions of HVR. These comparisons suggested that no single residue is responsible for the interaction with HIP2 and that there might be structural differences outside the HVR that also contribute to the HIP2 interaction.

Comparisons of 47 potyviruses showed that HVR is slightly hydrophilic and enriched in polar residues (Fig. 2 and Supplementary Fig. 2 in **II**). In most potyviruses, HVR contained either one or two negatively charged amino acids, namely glutamic acid (E) or aspartic acid (D). The next most frequent amino acids were the polar threonine (T) or serine (S) and the positively charged lysine (K). Small residues, including glycine (G) that allows flexible conformation within protein structure, and alanine (A), also occurred frequently within HVR. PSbMV HVR had two consecutive glycine (G) residues at positions 5-6.

Variability, the frequency of polar or charged residues, and the presence of the flexible, small residues, commonly indicate loop regions (Branden and Tooze 1999). In the structural predictions of HCpro made in the present study, the HVR was within a loop region that contained a β-turn (Fig. 5 in **II**). Loop regions sometimes participate in binding of a ligand or the formation of enzymatically active sites, and are themselves subject to conformational alterations. They are also critical for conformational flexibility of proteins, and may regulate the conformation of the protein and the accessibility of its active sites (Branden and Tooze 1999). Crystallization of the TuMV HCpro protease domain (aa 300-458) left the structure of a region containing HVR (aa300-335) undetermined (Guo et al. 2011), which indicates flexibility and variability of the structure in that area, or a requirement for interactions between the C-terminal and central domains for proper folding.

4.5.2 HCpro contains a conserved eIF4E binding motif (IV)

Some of the truncated forms of PVA HCpro were used to define the eIF(iso)4E-interaction domain in HCpro. YTHS mapping results were more ambiguous than those of HIP2 interactions and the regions of HCpro capable of conferring interactions with eIF(iso)4E seemed to be dispersed along its central and C-terminal domains (Fig. S4 in **IV**). The C-terminal domain along with the second half of the central domain of HCpro (aa 325-458) was evidently required for a strong eIF(iso)4E-interaction. A similar resolution has been achieved in studies showing that the central region of VPg of TuMV and LMV, and the C-terminal domain of CI of LMV, mediate interactions with eIF4E (Léonard et al. 2000, Roudet-Tavert et al. 2007, Tavert-Roudet et al. 2012).

The interactions of eIF4G or the other eIF4E-binding proteins occur via a linear 7 amino acid interaction module. The consensus of this 4EBD motif is YXXXXLΦ, where X is a variable amino acid and Φ is a hydrophobic residue (Rhoads 2009). PVA HCpro contained multiple sites that corresponded to a 4EBD motif: one at the NH_2-terminal domain, seven within the central domain, and two within the C-terminal domain. Comparison with other HCpro sequences by multiple alignment revealed that only two 4EBD candidate motifs were conserved, YINIFLA and YHAKRFF (amino acids 345 to 351 and 214 to 220, respectively), and both were predicted to fold into α-helical conformations. Within the YINIFLA motif, Ala351 was fully conserved while substitution of tyrosine (Y345) for histidine occurred in two, and substitution of leucine (L350) for phenylalanine occurred in six of the 47 potyviruses represented in the alignment. The YHAKRFF motif in the central part of HCpro was less conserved: for example, the tyrosine (Y214) was replaced by Ala, Arg, Lys, Ala, Ile or His in 13 potyviruses. The YINIFLA site was located in the C-proximal region of HCpro that was able to mediate a strong eIF4E-interaction in YTHS. Mutagenesis of this motif in HCpro of

PVA (substitutions of Y345 and L350 for alanine) reduced interactions with eIF4E and eIF(iso)4E (**IV**), showing that YINIFLA was a true 4EBD motif. Contribution of the other sites to the eIF4E-interaction is also possible.

An interesting exception in the 4EBD motif was observed in HCpro of TEV (**IV**): instead of L350 in the YINIFLA motif, all sequenced isolates of TEV had phenylalanine (F) resulting in sequence YMNIFFA. Mutagenesis studies on TEV HCpro coincidentally provide more information on the 4EBD motif candidates. A point mutation in HCpro of TEV replacing tyrosine for serine two amino acids before YINIFLA (Y344, aa 343 in PVA) resulted in milder symptoms and reduced virulence of TEV, whereas replacements of K218 and R219 (aa 217 and 218 in PVA) for alanine in the other conserved 4EBD candidate motif (YHAKRFF) did not significantly reduce TEV amplification and movement (Kasschau et al. 1997, Torres-Barceló et al. 2008). These results may indicate that neither the 4EBD nor the other candidate sites is necessary for pathogenicity of TEV. The C-proximal part of TEV HCpro, however, contained the sequence YLLSILY (residues 391 to 397), also corresponding to the consensus 4EBD motif. This site was conserved in all isolates of TEV but not in other potyviruses.

Secondary structure predictions and the 3D model of HCpro suggested that the 4EBD may adopt an α-helical conformation (**Fig. 4**, Fig. S3 in **IV** and Fig. 5 in **II**). Structural changes resulting in an α-helical fold are induced in the 4EBD motifs of the human eIF4G and the eIF4E-binding protein 4E-BP1 upon binding to eIF4E (Marcotrigiano et al. 1999). Similarly, a helix within the central domain of VPg of LMV may be involved in the VPg-eIF4E interaction (Roudet-Tavert et al. 2007).

Competition between VPg and mRNA cap structure for eIF(iso)4E has been suggested to occur in complex with eIF(iso)4G (Grzela et al. 2006). The putative additional 4EBD motifs in HCpro may signify the importance of HCpro-eIF4E interactions for virus infection and may provide an advantage for HCpro in the competition with other viral or host proteins.

4.5.3 Mutations in HVR but not in 4EBD alter the conformation of HCpro (II)

The HVR in the model of wild type (wt) HCpro was in a coil or loop structure and had a β-turn. The possible effects of mutations on the secondary and tertiary structure of HCpro were studied by predicting the conformations of the mutated HCpro proteins. The HVR adopted an α-helical conformation in the modelled structures of two of the HVR mutants that induced the severest reductions in HIP2 interactions. Further comparisons of HVR mutants suggested that the four types of HVR mutations all have a major impact on the conformation of the hinge domain (Fi. 5 in **II**). This result suggested that conformations of the hinge domain and HVR together may regulate HIP2 interaction, the conformation of HVR being the major determinant. All the mutations similarly affected the functional motifs related to RNA binding

(RNP-1) and systemic movement (CCC) (Cronin et al. 1995, Kasschau et al. 1997, Urcuqui-Inchima et al. 2000), both of which were within α-helices in the wtHCpro model but lacked a defined secondary structure in the models of HVR-mutants (**II**).

Provided that conformational transitions or switches occur commonly, e.g., in response to changes in environment or upon binding of a ligand, a single structural model is severely restricted as it represents only one conformation (Branden and Tooze 1999). In HCpro, the C-terminal protease domain could mask the central domain and regulate its accessibility, as suggested by Plisson et al. (2003). The flexibility of the hinge domain may allow different conformational shapes of HCpro for alternative functions. This is also implied by the dissimilarity of the quaternary structures of oligomers formed of HCpro of PPV or TEV in different crystallization environments (Plisson et al. 2003, Ruiz-Ferrer et al. 2005).

A 3D structure of the HCpro mutant with alanine substitutions in the 4EBD-motif (**IV**) was modelled as described for the HVR mutants (**II)** and compared with the wtHCpro model using the TM-align tool (Zhang and Skolnick 2005). A gapless alignment and distances less than 5Å between the backbone C_α atoms of the main chains of the two models indicated that the 4EBD mutations did not change the folding of HCpro (*unpublished results*). The normalized Tm score (Zhang and Skolnick 2004) of 0.99 also indicated a nearly perfect match between the models. In contrast, the similar 3D alignments between wtHCpro and the HVR-mutants (**II**) contained unaligned structures, particularly in the hinge region, and had lower TM scores (0.77-0.86). These results were in agreement with the role of the 4EBD motif as an interaction motif or pocket engaged in direct residue-to-residue interactions.

4.5.4 Three of the four HVR-mutants of HCpro are improved as silencing suppressors (III)

Mutations in multiple locations within any of the three domains of HCpro influence the efficiency of RNA silencing suppression of HCpro, impairment of which is correlated with reduced virulence and lower viral accumulation (Kasschau and Carrington, 2001, Varrelmann et al. 2007, Torres-Barceló et al. 2008). The tertiary conformation of HCpro may influence its efficiency as a suppressor because it would affect the availability of the residues in the central and C-terminal domains and therefore alter the potentially important interactions of HCpro with RNA or with HEN1 protein (Ballut et al. 2005, Varrelmann et al. 2007, Jamous et al. 2011). To study if the HVR mutations had pleiotropic effects on this critical function of HCpro, it was important to test if the HVR mutants retained their silencing suppression activity.

Silencing-on-the-spot is the standard method for assaying the silencing suppression ability of a protein in plant leaves and for comparing the relative efficiencies of different silencing suppressors (Johansen and Carrington

2001). In the present study, the HCpro proteins were expressed as fusions with a half of YFP, a variant of the jellyfish (*Aequorea victoria* Murbach and Shearer, 1902) green fluorescent protein (GFP), in their NH_2-terminal ends (**III**). NH_2-terminal fusions do not inhibit the suppression activity of HCpro though they may shorten the longevity of active suppression (Chiera et al. 2008). The intensity of GFP fluorescence and the analysis of the amounts of HCpro protein extracted from the infiltrated spots indicated that HCmA, HCmB and HCmC supported their own accumulation and that of *GFP* mRNA and GFP protein for a longer time than wtHCpro (Fig. 2 in **III**). No obvious differences in GFP intensity were observed between these three HVR mutants. The differences between the capacities of HVR mutants and wtHCpro to suppress silencing were similar, regardless of whether the silencing inducer was hairpin-*gfp* or a sense-*gfp* (*unpublished results*), but were easier to visualize in the case of sense-inducer because of the slower induction of silencing. These results showed that the three HVR mutants were more stable or better silencing suppressors for other reasons, but that the altered HIP2 interaction was not correlated with the improvement of the silencing suppression capacity.

In contrast to the other three HVR mutants, mutant HCmABC had only marginal suppression activity. This was correlated with very low HCmABC protein accumulation in leaves (Fig. 2 in **III**) and indicated that HCmABC was severely impaired as a suppressor.

Five TEV HCpro mutants with higher than normal suppression activity have been reported (Torres-Barceló et al. 2008). Three of them have an amino acid substitution within the C-terminal domain, one in the central domain (prior to the hinge region, aa 200) and one in the NH_2-terminal domain. The three hypersuppressor mutants with substitutions in the C-terminal domain bound to 21 nt siRNA less efficiently than wtHCpro, while binding efficiencies of the other two mutants did not significantly differ from wtHCpro (Torres-Barceló et al. 2010b). The molecular mechanism behind the enhanced suppression activities is not known. The silencing efficiency of HCpro is suggested to be correlated with the efficiency of its binding to siRNA species, with altered interaction with HEN1, or the balance between these (Torres-Barceló et al. 2010b, Jamous et al. 2011). Alternatively, apparent changes in suppression activity could be unrelated to RNA silencing suppression, and instead be due to changes in the interaction of HCpro with 20S proteasome regulating protein and RNA turnover (Ballut et al. 2005). The proteasome interactions are determined by NH_2-terminal domains of HCpro of PRSV and LMV (Dielen et al. 2011, Sahana et al. 2012) but the net effect of proteasome disruption on mRNA and protein accumulation remains to be determined.

These results indicated that amongst the mutants HCmA, HCmB and HCmC, the changes in the putative conformation of the hinge domain correlated with the RNA silencing suppression activity (**III**), while the folds of the hinge domain and HVR correlated with the strength of HIP2-

interaction (**II**). These observations also suggested that virulence experiments with the PVA HVR mutants, particularly comparisons of the fitness effects of the HCmB mutation with those of the other HVR mutations, could enlighten whether the HIP2-HCpro interaction has some role in PVA infection. Additionally, the HVR mutants improved and prolonged the expression of heterologous proteins in plants (**III**) which may have direct practical application in production of medical or other products in plants.

4.6 HIP2-HCpro interaction is required for efficient viral accumulation

4.6.1 Silencing of HIP2 and interference with HIP2-HCpro interaction reduce viral accumulation (I, II)

To address the question of the role of HIP2 and its interaction with HCpro in PVA infection, PVA accumulation was compared between *HIP2*-silenced plants and non-silenced plants (**I**), and accumulation of HVR mutants of PVA was compared with wtPVA (**II**). The accumulation levels of viruses depend on the efficiency of virus replication (a process involving translation and replication of viral genome), degradation of viral proteins or RNA in plant cells, and local movement. In order to study accumulation of PVA, leaves were inoculated by agroinfiltration. In this technique, multiple cells are rapidly and evenly transformed and infected with the virus, and the virus-infected regions can be sampled uniformly (Eskelin et al. 2010, **I**, **II**).

TRV-VIGS has been used to silence host factors or pathways involved in antiviral defence prior to infection with tobamovirus TMV (Liu et al. 2002, 2004) or potyviruses including PVA and TuMV (Rajamäki and Valkonen 2009, Yang et al. 2009, Hafrén et al. 2010). The silencing of *HIP2* in the *N. benthamiana* plants by using the TRV-VIGS showed that the amounts of PVA CP produced in inoculated areas were lower in silenced than in non-silenced plants (Fig. 3 in **I**).

When three types of HVR mutations that were not compromised for RNA silencing suppression (HCmA, HCmB and HCmC) were introduced to infectious clones of PVA and agroinoculated in *N. benthamiana* and tobacco leaves (Table 1 in **II**), all three accumulated significantly less than wtPVA, and HCmB tended to cause the severest reduction of PVA titers (Table 1 in **II**). A similar type of inoculation by agroinfiltration was previously used for PVA by Eskelin et al. (2010) in *N. benthamiana*. In the present study, the mutated viruses were compared pairwise to the wild type virus inoculated onto the opposite side of the midrib in the same leaf. This experimental design allowed exact pairwise comparison of each mutated virus to wild type virus and eliminated the effects of leaf age and tissue age, particularly within the large tobacco leaves. Leaves of potato are more difficult to infiltrate, and instead of agroinoculation, they were mechanically inoculated with

viruliferous leaf sap (**II**). Three weeks later, the inoculated leaves were collected and the accumulation of PVA was measured, giving sufficient time for the PVA to invade the inoculated leaf. The results were similar to those in *Nicotiana* species (Table 3 in **II**). Hence, the HIP2-interaction strengths of the mutated HCpro proteins correlated with the viral accumulation, but not with the silencing suppression activities of the same proteins.

In conclusion, a similar viral phenotype (slower accumulation) was caused by reducing the amounts of HIP2 in plants and by depleting HIP2 interaction with mutations in HCpro. HIP2 seems to be a host factor necessary for efficient accumulation of PVA, and a strong interaction between HIP2 and HCpro is associated with high accumulation.

The accumulation experiments were conducted in leaves, which may be important for normal functions of MTs. Virus accumulation measurements in protoplasts, on the other hand, require disruption of the cell wall for protoplast extraction. The fresh protoplasts soon engage in cell wall synthesis (Burgess and Fleming 1974), a process involving cortical MTs. Chemically induced disruption of the structures or interference with the dynamics of actin, MT or ER is also a commonly used tool in virus research, but potentially has profound effects on normal cellular functions. Mutated viruses with loss of interactions with these structures, or plants where specific genes are either knocked out or silenced, may be better suited for virulence studies (Harries and Ding 2011, Niehl et al. 2013).

The importance of the MT association for plant virus accumulation was a new discovery. The localization of PVA replication complexes at MT-associated sites (Fig. 4 in **I**) may be related to the requirement of HIP2 for virus accumulation (Fig. 3 in **I**). MT interactions of plant viruses are indicated in cell-to-cell movement and transmission of viruses, or in degradation of viral proteins (reviewed in Nieh et al. 2013), but there is no report of a direct role of MTs in virus accumulation. The requirement of HIP2 for virus accumulation might be unique in potyviruses, or represent a common mechanism of employing MT intersections or nucleation sites (in plants) and MTOC (in animal cells) for gathering and recruitement of cellular resources. Indeed, the MT and ER interactions of TMV MP may have multiple roles in TMV movement and multiplication, e.g., in the creation of sites important for the formation of replication or movement complexes and in regulation of the availability of MP in them (Niehl et al. 2013).

4.6.2 HVR mutations retard the initiation of systemic infection by PVA (II)

MT-interactions of plant viruses often occur with movement-related proteins and are implicated in virus movement, so it was relevant to study the effects of depletion of HIP2 or HIP2-HCpo interaction on virus movement. This was achieved in experiments involving *HIP2*-silenced plants (**I**) and HVR-mutated PVA (**II**), as in the experiments studying virus accumulation.

Systemic movement of GFP-tagged PVA was observed at almost the same time (within a few hours) in the upper leaves of *HIP2*-silenced and non-silenced control plants. This result indicated that reduction of HIP2 did not greatly affect the systemic movement. Minor differences in the unloading of PVA into systemically infected leaves (Fig. 3C,D in **I**) suggested that either infection or movement of PVA was initiated more slowly in *HIP2*-silenced plants.

The infectious PVA constructs carrying the HVR substitutions were sometimes detected later than the wtPVA in systemically infected leaves of *N. benthamiana* and potato (Table 2 and 3 in **II**). Particularly in potato, the delay of systemic infection was clearer and mutated viruses were detected in upper leaves only 3-4 weeks post inoculation while wtPVA was detected consistently already 2 weeks post inoculation (**II** and *unpublished results*). In addition, the mutated viruses were detected in lower parts of the potato and tobacco plants than the wild type virus that invaded younger leaves or leaflets that were not yet fully expanded (*unpublished observations*).

These results suggested that the lower accumulation of HVR mutants reduced their capacity to move systemically. Reduced viral accumulation could directly reduce the ability of a virus to invade plants, a phenomenon that may have relevance in crop protection in the field. It is also possible that a separate defence mechanism protected plants against systemic infection.

4.6.3 HVR mutation HCmABC does not prevent replication and movement of PVA (II, III)

The HVR mutant HCmABC, in which all the amino acid residues of HVR were mutated, did not interact with HIP2 in YTHS (Fig. 2 in **II**), but in contrast to the other HVR mutants, it was hardly functional as a RNA silencing suppressor (Fig. 2 in **III**). When tested with enzyme-linked immunosorbent assay (ELISA), PVA with this mutation (PVAmABC) did not accumulate to detectable levels in inoculated leaves of *N. benthamiana*, and was detected in upper leaves of only a few plants at low virus titers at 3 weeks after inoculation (**II**). ELISA testing of tobacco leaves agroinoculated with the mutated virus produced low but consistently elevated absorbance values as compared to non-replicative control virus (*unpublished results*). These results showed that PVAmABC could replicate and move systemically, although its fitness was heavily reduced.

The main cause of the reduced accumulation and virulence of PVAmABC may be its severely compromised RNA silencing suppression ability. Nevertheless, the ability of PVAmABC to replicate and move systemically suggested that interaction with HIP2 was not absolutely required for movement or replication.

4.7 Necrotic symptoms in *Nicotiana* spp. induced by HVR mutants of PVA

4.7.1 HVR mutants are hypervirulent in *Nicotiana* spp. (II)

No clear differences in symptoms were observed at the initial stages of infection in *N. benthamiana* and tobacco inoculated with wtPVA or the HVR mutants PVAmA, PVAmB and PVAmC. Also systemic infection both with the wtPVA and HVR mutants caused systemic mosaic symptoms and, in *N. benthamiana*, leaf malformation 2 weeks post-inoculation, although in plants infected with PVAmB the malformation symptoms were milder. However, the severity of the symptoms induced by the HVR mutants increased with time, and necrotic symptoms appeared in the top leaves and side shoots of *N. benthamiana* infected with the HVR mutants, but not in those infected with wtPVA (*unpublished results*, **Fig. 6**). Necrosis sometimes reached the apical meristems of either the main or the side shoots. The severity of these symptoms correlated with the virus titers of the HVR mutants, PVAmA and PVAmC causing more visible necrosis than PVAmB that accumulated less (Table 2 in **II**).

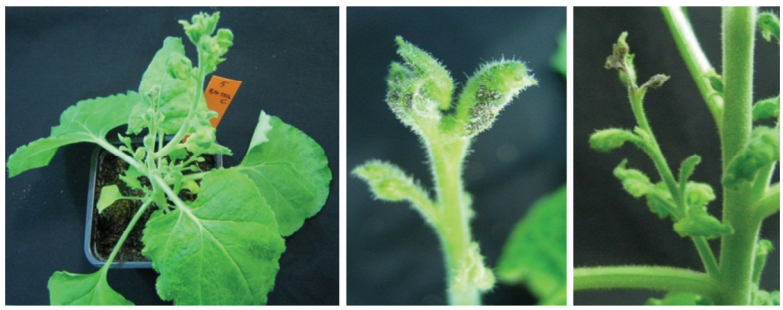

Figure 6 Necrotic symptoms in a *Nicotiana benthamiana* plant infected with PVAmC. Necrosis was observed in tip leaves on the main stem (middle) and side shoots (right) 3 weeks post-inoculation. The whole plant is shown to the left.

In tobacco, wtPVA typically induced only mild and temporary symptoms in the beginning of systemic infection (8-10 dpi), visible as chlorotic spots (Fig. 3B in **II**) or mild vein clearing in the systemically infected leaves. In contrast, persistent chlorotic spots or strong veinal chlorosis was observed in plants infected with the HVR mutants (PVAmA, PVAmB and PVAmC). Chlorotic symptoms coincided with the appearance of virus in the upper leaves. Observation under ultraviolet A (UV-A) light revealed blue fluorescence within the chlorotic lesions of systemically infected leaves 12-15 days post-inoculation (Fig. 3A in **II**). Experiments with GFP-tagged viruses demonstrated that the blue autofluorescence was initially visible in the

centres of infection foci (Fig. 3C **II**) wherein cells were lysed (*unpublished observations*). The chlorotic lesions turned to brown colour during the following 3-7 days, and subsequently concentric necrotic rings were formed (Fig. 3B **II**). The severest necrotic symptoms developed in the oldest systemically infected leaves. Tobacco plants infected with the HVR mutants of PVA were smaller and flowered later than those infected with wtPVA, indicating that the defence reactions interfered with their growth (*unpublished observations*). Mosaic symptoms (diffuse chlorotic mottling) commonly observed in the top leaves of wtPVA-infected tobacco at 3-5 weeks post-inoculation were absent in plants infected with HVR mutated viruses.

The necrotic symptoms were novel for PVA, not having been observed previously in tobacco or *N. benthamiana* plants infected with any of the PVA strains or their chimeras (Rajamäki et al. 1998, Valkonen et al. 2002, Paalme et al. 2004). Compared to wtPVA, the HVR mutants were hypervirulent. Even the mutant PVAmB, which was less virulent than the two other HVR mutants, caused this hypervirulent reaction in *Nicotiana* spp.

Necrotic symptoms caused either by pathogen or by HR-inducing elicitors are often preceded by expression of fluorescent compounds, and associated with induction of localized acquired resistance (LAR) in tobacco (Dorey et al. 1997, Costet et al. 2002). The blue-green autofluorescence has been used to detect initiation of HR to TMV in tobacco, in which case the fluorescent compound is probably scopoletin (Mock et al. 1999, Chaerle et al. 2007) and to detect asymptomatic systemic infection of *Pepper mild mottle virus* in *N. benthamiana*, in which the fluorescing compound might be chlorogenic acid (Pineda et al. 2008). Chlorogenic acid (3-caffeoylquinic acid) and scopoletin (7-hydroxy-5-methoxycoumarin) are stress-inducible antioxidant compounds of the phenylpropanoid pathway. Antioxidant properties of scopoletin slightly attenuate HR (Costet et al. 2002). HR alone is not sufficient to arrest the virus in infected tissue, as known for other virus-plant interactions (Dinesh-Kumar et al. 2000, Hamada et al. 2005), and the LAR is often succeeded by induction of systemic resistance in the whole plant, e.g., an SA-dependent SAR.

Although the antimicrobial secondary metabolites are targeted against extracellular pathogens, they may directly or indirectly work against virus infections. For example, phenylpropanoid accumulation is involved in confinement or reduction of PVY in systemically infected leaves of tobacco (Matros et al. 2006).

4.7.2 Hypotheses of the mechanisms of necrosis induction

Defence reactions in plants depend on recognition of either general molecular patterns associated with pathogens or damage, or the more specific R-gene mediated recognition of pathogen effectors, and on further amplification of defence signals (Jones and Dangl 2006). The HVR mutations altered the HIP2 interaction, the capacity to suppress RNA

silencing, and the conformation of HCpro, any of which may have caused the altered pathotypes of the HVR-mutated PVA (**Table 2**). The reduced fitness of PVA HVR mutants was detected in both *Solanum* and *Nicotiana* species, but necrosis was observed only in the species of genus *Nicotiana*. This indicated that the necrotic response might be separate from the restriction of viral accumulation.

Table 2. *Summary of the effects of the mutations in the highly variable region (HVR) of HCpro on the conformation and functions of HCpro protein and on the accumulation and virulence of PVA in* Nicotiana *spp.*

	no. of aa[a] changes	Modelled protein conformation in		Interaction in YTHS[c]		RSS[d]	Virus titer	Necrosis
HCpro		HVR[b]	hinge	HIP2	Self			
wt	-	wt	wt	+++	+++	++	+++	no
HCmA	1	wt	altered	++	+++	+++	++	yes
HCmC	3	wt	altered	++	+++	+++	++	yes
HCmB	4	altered	altered	+	+++	+++	+	yes
HCmABC	6	altered	altered	-	+++	(+)	-	no

[a] amino acid; [b] highly variable region; [c] yeast two-hybrid system; [d] RNA silencing suppression

Three of the four HVR-mutants of HCpro were hypersuppressors (**III**). The reduction in RNA silencing suppression capacity of HCpro of TEV is correlated with low accumulation and virulence of the virus (Torres-Barceló et al. 2008). However, the bearing of hypersuppressor mutations on viral fitness or virulence, if any, is not straightforward. Five hypersuppressor mutants of HCpro of TEV have been reported, one of which reduced virus accumulation while the other four had no significant effect on virus accumulation or virulence (Torres-Barceló et al. 2008).

Necrosis triggered by systemic infection with HVR mutants of PVA could have been induced by the changed conformation of the hinge part of HCpro (**II**). The altered conformation was optimal for RNA silencing suppression (**III**), but may be it was not suitable for other defence-suppression functions by HCpro. Alternatively, the conformational change itself may have exposed HCpro to direct recognition by the host, particularly as the changes may have exposed an RNA binding region of HCpro that are typical for the viral proteins. Nevertheless, no adverse effects were visible on leaf tissues upon overexpression of HVR-mutated HCpro proteins in the absence of infection, despite their prolonged expression and high accumulation levels (Fig. 2 in **III**). Elicitors that are recognized by an R gene, commonly trigger a diagnostic HR-like response in leaves in the absence of pathogen (Baillieul et al. 1995). Individual viral proteins can also trigger local HR, as shown by expression of TMV CP, or the helicase domain of TMV replicase, which are

recognized by the R proteins N' or N, respectively (Culver and Dawson 1991, Abbink et al. 1998). In some cases, however, an R-gene mediated recognition is enabled only along with viral movement (Canto and Palukaitis 1999). The necrotic responses to PVA HVR mutants were detected only in the systemically infected parts of *Nicotiana* spp., implicating a recognition of a process in the virus infection cycle rather than a direct recognition of HCpro conformation.

The present work allows the formulation of four hypotheses for the initiation of necrotic symptoms in *Nicotiana* plants infected by PVA HVR mutants.

A. HCpro structure is changed

1. **ETI hypothesis**: HCpro is recognized directly by an unknown *R* gene because the altered conformation exposes a recognizable area within HCpro.
2. **Effector – PTI hypothesis**: The general cellular changes related to viral infection are recognized because they are not shielded by the mutated HCpro.

B. HCpro-HIP2 interaction is reduced

3. **MT-localization hypothesis**: targeting of the mutated HCpro to MTs is reduced and HCpro-MT interactions and their functions (e.g., HCpro-induced reorganization of transport) are cancelled, which triggers recognition and activation of ETI or PTI.
4. **Signalling hypothesis**: HIP2 may be a sensor or integrator of signalling pathways. The weakened interaction of HIP2 and the mutated HCpro is unable to interrupt or alter the defence signalling.

4.7.3 The necrosis is induced SA-independently (II)

Transgenic expression of HCpro in plants interferes with both SA-dependent and SA-independent defences (Pruss et al. 2004), and there is an interaction between the HCpro-mediated RNA-silencing suppression and the SA-mediated host responses against potyviruses (Alamillo et al. 2006, Atsumi et al. 2009). To study whether the SA is required for the necrotic symptoms caused by the PVA HVR mutants, transgenic tobacco plants that express salicylate hydroxylase (NahG) to degrade SA (Friedrich et al. 1995) were inoculated with wtPVA and PVA HVR mutants. Similar necrotic symptoms were induced in both *NahG*-transgenic and non-transgenic plants infected with HVR mutants (Supplementary Fig. 4 in **II**), indicating that SA-mediated signalling was not required for the response.

SA-induced defences are not always efficient against viruses, but SA-related signalling can modify the necrotic phenotypes of viral diseases (Huang et al. 2005, Love et al. 2005). Two kinds of effects have been observed in different potyvirus infections: SA reduces and delays necrotic symptoms in the PVY – tobacco pathosystem based on an *R* gene, while it

enhances symptoms in garden pea (*Pisum sativum* L.) infected with ClYVV (Nie 2006, Atsumi et al. 2009). In the case of HVR mutants of PVA, the influence of the SA-mediated pathway during later stages of systemic infection remained possible, and systemic signalling and SAR may have restricted infection after the induction of necrosis, as observed by Alamillo et al. (2006). However, that aspect was not analysed in this thesis.

4.7.4 Gene expression in HVR-induced defence response (II)

To study the types of defence-related pathways and processes induced before the appearance of visible necrosis, gene expression in tobacco leaves infected with the HVR mutant PVAmC was compared with the leaves infected with wtPVA using tobacco microarray. A single time point just prior to appearance of visible necrosis in systemically infected leaves was studied, which potentiated a large-scale analysis of gene expression at that time, but not time-course or spatial analyses of the responses.

As expected, genes involved in defence responses, especially genes of JA and ET signalling pathways and ER stress, were induced in response to infection with HVR mutants, while SA-related genes were less induced (Fig. 4. in **II**). The induction of SA-signalling and related PR genes has been previously associated with infection by compatible viruses (Witham et al. 2003). Activation of the JA pathway increases secondary metabolites and reduces growth by antagonizing GA synthesis (Reinbothe et al. 1994, Heinrich et al. 2012, Yang et al. 2012). The transcription of genes for growth and energy metabolism, including photosynthesis, was repressed, as is also observed in compatible virus infections (Yang et al. 2007). These results were consistent with the observed reduction in growth in plants infected with the hypervirulent HVR mutants. The altered expression of genes involved in secretion and upregulation of secondary metabolism were in agreement with the phenotypic observations of accumulation of phenolic compounds. Interestingly, many MT-related genes, including kinesins, were downregulated.

The expression of HCpro of PPV from a heterologous PVX virus vector in *N. benthamiana* plants is accompanied with systemic necrosis, upregulation of MT-related genes, and other changes in gene expression which are similar to those observed in plants silenced for proteasome genes (Pacheco et al. 2012). The downregulation of the MT-associated genes in the present study suggest that the necrotic responses caused by the PVA HVR mutants may be dissimilar to the response induced by the PPV HCpro, and unrelated to proteasome dysfunction, or to the enhanced silencing suppression capacity of HVR-mutated HCpro.

Expression of *HIP2* among the MT-associated genes was not significantly changed, but interestingly, the expression of the gene for a homolog of RLK902 and RLK1, that are SPR2-interacting proteins in *Arabidopsis*, was upregulated. The genes for *RLK902* and *RLK1* in *Arabidopsis* are

downregulated in infection with compatible or incompatible *Pseudomonas syringae* pv. *maculocola* strains and show temporal downregulation when plants are treated with SA or wounded (Tarutani et al. 2004b).

The HR marker gene *HSR203J* (Pontier et al. 1998) was induced, indicating that the observed response was related to hypersensitive response (ETI) rather than PTI. Genes related to autophagy, which is needed to restrict HR-associated cell death (Patel and Dines-Kumar 2008), were not induced.

In conclusion, the gene expression analysis confirmed that the observed response appeared to be an SA-independent defence response resulting in HR, which would indicate that an ETI-type defence was triggered. These results could not, however, show whether the cell death was activated by an R-protein based recognition, leading to a signalling pathway, or because a defence signal was transduced or amplified in a different way.

4.7.5 A model of HIP2 in signalling

As explained in the section 1.5.3, three other studies have reported interactions of HIP2-homologous proteins from *Arabidopsis*, tomato and sugar beet (Mukhtar et al. 2011, Ben-Naim et al. 2007, Thiel and Varrelmann 2009). All of these interactions may be related to developmental regulation or defence signalling, as they include transcription factors and receptor-like kinases that interact with *Arabidopsis* SPR2 or tomato HIP2, and a viral protein that interacts with a HIP2-homolog of sugar beet. These observations could indicate that HIP2 or SPR2 is an integrator of several signalling networks in the development and sensing of stresses. That kind of role is suitable for the structural flexibility of alpha-solenoid proteins, that are often engaged in several interactions and consequently may act as adaptor proteins (Flynn 2001, Kappel et al. 2010). Indeed, a receptor-like kinase TARK1 from tomato, which is a putative interaction partner of HIP2, is targeted by and interacts with a *Xanthomonas* effector XopN that is an alpha-solenoid protein (Kim et al. 2009). The authors proposed that this interaction replaces a signalling scaffold protein of the host, allowing the XopN effector to modulate RLK-dependent signalling to promote virulence of the bacteria. Consistent with the scaffold hypothesis, TARK1 and a component of a presumably unrelated signalling pathway, TFT1, form complexes in the cortical cytoplasm or the PM of plant cells in the presence of XopN but not in its absence (Taylor et al. 2012).

MT intersections, MT ends and γ-tubulin complexes along the MT array have components that interact with membranes, indicating that the localization pattern of HIP2 would be ideal for interactions with the membrane-associated receptors and endosomes. For these interactions, HIP2 might also interact with the MT (+)end scaffold protein EB1 and collaborate with CLASP. The regulation of dynamics or organization of MTs by MT-associated proteins may directly affect defence signal transduction

(Shi et al. 2009, Qiao et al. 2010, Yao et al. 2011) and the related endosomal traffic (Ambrose et al. 2013).

HCpro interaction with the AHR domain of HIP2 could affect the self-interaction or the other heterologous interactions of HIP2, its spatial distribution, and its functioning in MT dynamics. MTs are at the centre of actin-propelled cytoplasmic traffic and mark the cortical sites where cell organelles and RNA-bodies pause for putative cargo recycling, exchange, or secretion (Hamada et al. 2012). These are optimal sites for resource allocation for a virus. Hence, HCpro might target HIP2 to influence the cellular organization and the logistics of the resource distribution via regulating MTs. Simultaneously, HCpro could target HIP2-associated protein complexes in defence or developmental signalling.

5 CONCLUSIONS AND FUTURE PERSPECTIVES

The present study characterized interactions of HCpro with two host factors. The translation-related protein, eIF4E, is a known host factor involved in the recessive resistance to potyviruses that inhibits virus multiplication or systemic movement. Silencing of the MT-associated protein HIP2 demonstrated that it also is a host factor and required for efficient accumulation of PVA. Together with the subcellular localizations of the interactions, the data suggested that potyviral HCpro is involved in viral replication or accumulation (**Fig. 7**), perhaps independent of its role in suppression of RNA silencing.

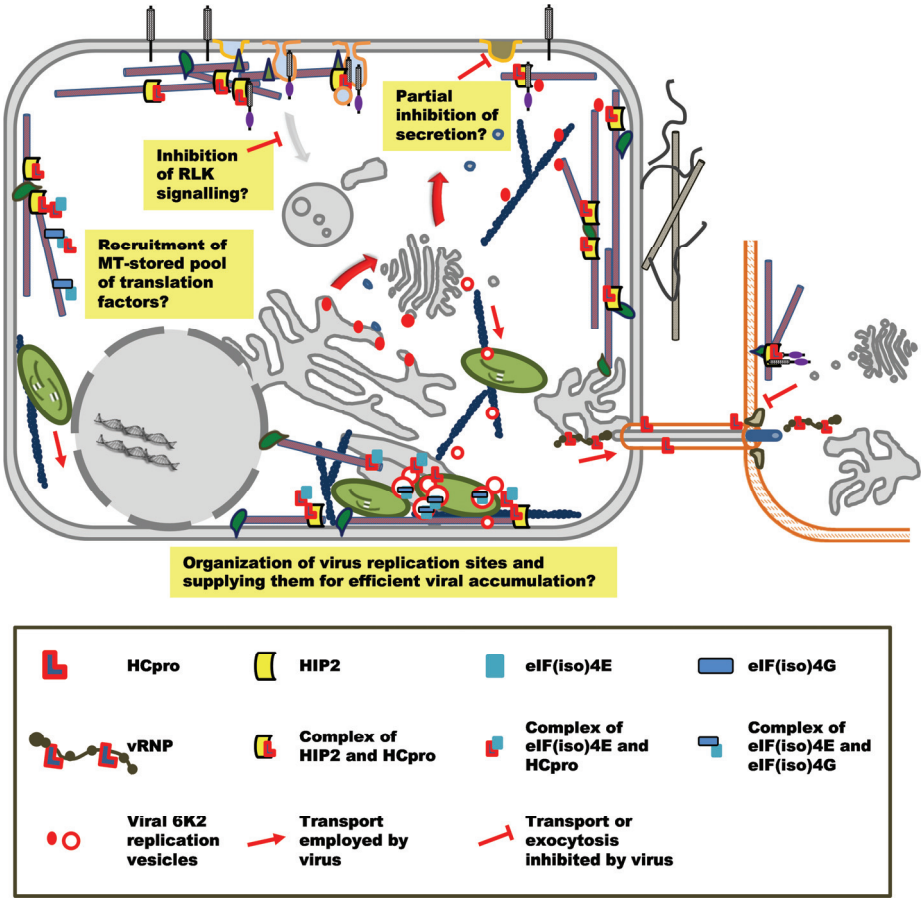

Figure 7 Schematic drawing of the localizations of potyviral HCpro in an infected cell and putative functions of the MT- and replication vesicle-associated localizations. MT, microtubule; HCpro, helper component proteinase; HIP2, HCpro-interacting protein 2; eIF(iso), isoform of eukaryotic initiation factor 4E or 4G; 6K2, viral 6 kilodalton protein; vRNP, viral ribonucleoprotein complex; RLK, receptor-like kinase. The other symbols are as in Fig. 2.

Depletion of HIP2 interaction with HCpro mutagenesis reduced PVA accumulation but also caused a hypervirulent, necrotic infection phenotype. Hence, interaction of HCpro with HIP2 might be involved in MT-mediated modification of the subcellular environment, possibly in accumulation or targeting of host resources for viral replication. It could additionally be the means by which the virus supresses defence signalling or defence responses (Fig. 7).

A possible scenario that combines the MT association and eIF4E interactions of HCpro is that HCpro may recruit or sequester specific MT-tethered translation or RNA modification factors from MTs. HIP2 or HIP2-associated antiviral receptors would guard these factors. By interacting with HIP2, HCpro would intercept HIP2 from forming signalling-associated complexes in the cell cortex or releasing such to be transported to the nucleus or other parts of the cell. To elucidate this hypothesis, host or viral proteins in same complexes with HCpro and HIP2 should be found and identified.

Despite the major differences in interphase MT organization between the plant kingdom and the other eukaryotes, the conserved eukaryotic multi-TOG-domain proteins MAP215 and CLASP are functional in plants. The results presented in this thesis suggested that the HIP2 and SPR2 proteins are putative new TOG domain proteins. They may be needed for MT dynamics or signalling events in the cortical MT array. Testing the ability of the putative TOG domain in HIP2 to bind tubulin heterodimers would be an important part of any further analysis.

Studies on the molecular mechanisms of the cellular functions of HIP2 were initiated in the present study. Self-interactions of HIP2 studied in this thesis helped to elucidate its putative similarity to the conserved TOG-domain proteins that, in contrast to HIP2, contain multiple TOG domains. The spatial distribution of HIP2 between the dynamic MT (+)-ends, MT lattice and MT intersections (putative cortical MT nucleation sites) may be affected by the self-interactions of HIP2. That, in turn, may be a mechanism regulating HIP2 functions. Elucidation of the interactions of HIP2, SPR2, and of their homologs *in planta* would cast light on the role of HIP2 in cellular signalling and responses. The spatial distribution of the HIP2-interaction partners could be studied to find out if they are, e.g., transported to the nucleus for signalling. The role of HIP2 and MTs in stress responses would be best determined by using transgenic plants with a fluorescently tagged HIP2 expressed at natural levels.

Two testable hypotheses of how HCpro might interfere with an MT-mediated cellular signalling at molecular level are

a) by (selectively) inhibiting other interactions of HIP2, and

b) by regulating the localization of HIP2 within the MT array.

The results of the present study may help us to understand how viruses that have a high fitness but cause only minor symptoms (low virulence) interact with their host. It is interesting to speculate that if new alleles of

HIP2 or eIF4E could be found that have weaker or no interactions with HCpro of PVA, whether those could be used as resistance traits in restriction of viral accumulation. The recessive eIF4E-based resistance traits are commonly used in resistance breeding. Interactions of HCpro of other potyviruses, or their strains, with the HIP2 and eIF4E proteins of their host and non-host plants should be explored on a larger scale to evaluate the applicability of these genes in resistance breeding of potato. In the case of HIP2, both non-interacting and strongly interacting alleles could be interesting as variants with strong HCpro interaction could potentially attenuate aggressive strains of potyviruses.

MT-related traits are also involved in cold hardiness and salt tolerance, both critically important in plant breeding in a changing climate. The MT-related resistance or tolerance traits may be associated with altered reactions to pathogen infection or adverse conditions. They may also be affected differently by regular agricultural practices, e.g., upon application of MT-targeting herbicides, or in combination of adverse conditions and herbicide application. Hence, such possibilities should be explored prior to practical introduction of MT-based resistance or tolerance traits in plants.

Acknowledgements

It takes much more than the efforts of a PhD candidate to complete a thesis. I am grateful to my supervisors, Professor Jari Valkonen and Docent Minna Rajamäki for their guidance, optimism and trust, and that they have given me enough freedom to experiment and develop, sometimes the hard way. Their attitude to science and conduct as scientists has shown me how to progress and keep one's goals clear. The pre-examiners, Docent Kirsi Lehto and Professor Ykä Helariutta, are thanked for their critical and encouraging comments. Professor Helariutta and Nisse Kalkkinen, as the members of my follow-up group, are thanked for their time, valuable suggestions and interest in my research.

I wish to thank my co-authors and other colleagues who have collaborated during these years. Marjo, Elisa, and Tian, your input and determined work have been critical to transform the results into publications. Many people have shown me techniques, given me protocols, or brainstormed with me, including: Anssi (so many protocols), Xianbao, Tian (with whom I share the passion for the structure of the multitasker HCpro), Satu Lehesranta from professor Helariutta's group, Isa, Jani, Mikko, Anneke, Johanna N, Sascha, Olga and Johanna S. Panu Somervuo did the statistical analysis of the microarray data and Mikko helped me to get started with the analysis of the data. I am also in debt for the materials and other practical help from Xianbao, Sascha, Mikko, Shahid, Anssi, Sidona and Minna Rännäli. I am very glad to have had Anni Piippo's help in some of my lab work, which she conducted with energy, efficiency, and interest.

This research was conducted in the Department of Agricultural Sciences, Faculty of Agriculture and Forestry. I would like to thank the professors and lecturers already from the time of my undergraduate studies, who helped me to understand the benefits and promises of molecular methods and gene technology in agricultural sciences. I owe much to my MSc thesis supervisor, Professor Teemu Teeri, and his group for letting me in and guiding me in many techniques of molecular laboratory. I wish to thank the professors and docents Paula Elomaa, Minna Pirhonen, Minna Haapalainen, Anna Kärkönen, Timo Hytönen, Kristiina Himanen, Fred Stoddard and Mervi Seppänen and their group members for various good interactions during these years.

The facilities and work of the technical staff in the department and in Viikki campus have been valuable. The personnel of the Light Microscopy Unit in the Institute of Biotechnology (Mika Molin, Kimmo Tanhuanpää, Marco Crivaro) is thanked for the excellent technical support and advice. Funding from the Finnish Doctoral Program in Plant Sciences, and the funding that Professor Valkonen obtained and distributed for my work, are all appreciated. Karen Sims-Huopaniemi as coordinator of the doctoral

program has created the frame and spirit for interaction for students from various departments and universities. Dr Fred Stoddard is thanked for the language revision of this thesis, but the author wishes to take the blame for any mistakes remaining.

As a member of a large department, I have had the pleasure of the company of numerous current and former students. To mention a few, Anu, Johanna N, Marja, Tommaso, Merete, Elisa G, Elisa S, Marcelina, Sascha, and the lunch-team with Anssi, Johanna, Mikko, Jani and others: you have lightened the everyday. Isa, thank you for always taking up the slack, and the pleasant distractions! Kati and Martin, thank you for providing the much-needed distraction in the form of books in plenty!

Occasionally during these years I may have made my family, relatives and friends feel neglected. I have learned of their patience and love from their sharing the frustration and joy, their questions, and their kind understanding of sometimes not making many of those. Heartfelt thanks are for my parents, who have induced the interest in learning and reading to compensate the hard work, and for my husband Ville, who cares to remind me of the broader picture, both in science and in life.

REFERENCES

Abbink TEM, Tjernberg PA, Bol JF, Linthorst HJ. 1998. *Tobacco mosaic virus* helicase domain induces necrosis in *N* gene-carrying tobacco in the absence of virus replication. *Molecular Plant-Microbe Interactions* 11, 1242–1246.

Acosta-Leal R, Xiong Z. 2008. Complementary functions of two recessive R-genes determine resistance durability of tobacco 'Virgin A Mutant' (VAM) to *Potato virus Y*. *Virology* 379, 275–283.

Adams MJ, Antoniw JF, Fauque CM. 2004. Molecular criteria for genus and species discrimination within the family *Potyviridae*. *Archives of Virology* 150, 459–479.

Afzal AJ, Wood AJ, Lightfoot DA. 2008. Plant receptor-like serine threonine kinases: roles in signaling and plant defense. *Molecular Plant-Microbe Interactions* 21, 507–517.

Akhmanova A, Steinmetz MO. 2008. Tracking the ends: a dynamic protein network controls the fate of microtubule tips. *Nature Reviews. Molecular Cell Biology* 9, 309–322.

Al-Bassam J, Chang F. 2011. Regulation of microtubule dynamics by TOG-domain proteins XMAP215/Dis1 and CLASP. *Trends in Cell Biology* 21, 604–614.

Al-Bassam J, Kim H, Brouhard G, van Oijen A, Harrison SC, Chang F. 2010. CLASP promotes microtubule rescue by recruiting tubulin dimers to the microtubule. *Developmental Cell* 19, 245–258.

Al-Bassam J, Larsen NA, Hyman AA, Harrison SC. 2007. Crystal structure of a TOG domain: conserved features of XMAP215/Dis1-family TOG domains and implications for tubulin binding. *Structure* 15, 355–362.

Al-Bassam J, van Breugel M, Harrison SC, Hyman A. 2006. Stu2p binds tubulin and undergoes an open-to-closed conformational change. *The Journal of Cell Biology* 172, 1009 –1022.

Alamillo JM, Saénz P, García JA. 2006. Salicylic acid-mediated and RNA-silencing defense mechanisms cooperate in the restriction of systemic spread of Plum pox virus in tobacco. *The Plant Journal* 48, 217–227.

Albrecht V, Šimkova K, Carrie C, Delannoy E, Giraud E, Whelan J, Small ID, Apel K, Badger MR, Pogson BJ. 2010. The cytoskeleton and the peroxisomal-targeted SNOWY COTYLEDON3 protein are required for chloroplast development in Arabidopsis. *The Plant Cell* 2, 3423-3438.

Ambrose C, Ruan Y, Gardiner J, Tamblyn LM, Catching A, Kirik V, Marc J, Overal R, Wasteney GO. 2013. CLASP interacts with sorting Nexin 1 to link microtubules and auxin transport via PIN2 recycling in *Arabidopsis thaliana*. *Developmental Cell* 24, 649–659.

Ambrose JC, Shoji T, Kotzer AM, Pighin JA, Wasteneys GO. 2007. The Arabidopsis *CLASP* gene encodes a microtubule-associated protein involved in cell expansion and division. *The Plant Cell* 19, 2763–2775.

Anandalakshmi R, Pruss GJ, Ge X, Marathe R, Mallory AC, Smith TH, Vance VB. 1998. A viral suppressor of gene silencing in plants. *Proceedings of the National Academy of Sciences of the United States of America* 95, 13079–13084.

Andrade MA, Bork P. 1995. HEAT repeats in the Huntington's disease protein. *Nature Genetics* 11, 115–116.

Andrade MA, Petosa C, O'Donoghue SI, Müller CW, Bork P. 2001. Comparison of ARM and HEAT protein repeats. *Journal of Molecular Biology* 309, 1–18.

Andrejeva J, Puurand Ü, Merits A Rabenstein F, Järvekülg L, Valkonen JPT. 1999. Potyvirus helper component-proteinase and coat protein (CP) have coordinated functions in virus-host interactions and the same CP motif affects virus transmission and accumulation. *Journal of General Virology* 80, 1133 –1139.

Ashby J, Boutant E, Seemanpillai M, Sambade A, Ritzenthaler C, Heinlein M. 2006. *Tobacco mosaic virus* movement protein functions as a structural microtubule-associated protein. *Journal of Virology* 80, 8329- 8344.

Atsumi G, Kagaya U, Kitazawa H, Nakahara KS, Uyeda I. 2009. Activation of the salicylic acid signaling pathway enhances *Clover yellow vein virus* virulence in susceptible pea cultivars. *Molecular Plant-Microbe Interactions* 22, 166–175.

References

Ayaz P, Ye X, Huddleston P, Brautigam CA, Rice LM. 2012. A TOG:αβ-tubulin complex structure reveals conformation-based mechanisms for a microtubule polymerase. *Science* 337, 857–860.

Baillieul F Genetet I, Kopp M, Saindrenan P, Fritig B, Kauffmann S. 1995. A new elicitor of the hypersensitive response in tobacco: a fungal glycoprotein elicits cell death, expression of defence genes production of salicylic acid, and induction of systemic acquired resistance. *The Plant Journal* 8, 551–560.

Ballut L, Drucker M Pugniere M Cambon F Blanc, S Roquet F Candresse T, Schmid H-P, Nicolas P, Le Gall O, Badaoui S. 2005. HcPro, a multifunctional protein encoded by a plant RNA virus, targets the 20S proteasome and affects its enzymic activities. *Journal of General Virology* 86, 2595–2603.

Baluška F, Šamaj J, Wojtaszek P, Volkmann D, Menzel D. 2003. Cytoskeleton-plasma membrane-cell wall continuum in plants. Emerging links revisited. *Plant Physiology* 133, 482–491.

Barnard DC, Cao Q, Richter JD. 2005. Differential phosphorylation controls Maskin association with eukaryotic translation initiation factor 4E and localization on the mitotic apparatus. *Molecular and Cellular Biology* 25, 7605–7615.

Barton DA, Overall RL. 2010. Cryofixation rapidly preserves cytoskeletal arrays of leaf epidermal cells revealing microtubule co-alignments between neighbouring cells and adjacent actin and microtubule bundles in the cortex. *Journal of Microscopy* 237, 79–88.

Barton DA, Vantard M, Overall RL. 2008. Analysis of cortical arrays from *Tradescantia virginiana* at high resolution reveals discrete microtubule subpopulations and demonstrates that confocal images of arrays can be misleading. *The Plant Cell* 20, 982- 994.

Baulcombe DC. 2007. Amplified silencing. *Science* 315, 199–200.

Beauchemin C, Boutet N, Laliberté J-F. 2007. Visualization of the interaction between the precursors of VPg, the viral protein linked to the genome of *Turnip mosaic virus*, and the translation eukaryotic initiation factor iso 4E *in planta*. *Journal of Virology* 81, 775–782.

Beauchemin C, Laliberté J-F. 2007. The poly(A) binding protein is internalized in virus-induced vesicles or redistributed to the nucleolus during *Turnip mosaic virus* infection. *Journal of Virology* 81, 10905–10913.

Bedhomme S, Elena SF. 2011. Virus infection suppresses *Nicotiana benthamiana* adaptive phenotypic plasticity. *PLoS One* 6, e17275.

Bendahmane A, Kanyuka K, Baulcombe DC. 1999. The *Rx* gene from potato controls separate virus resistance and cell death responses. *The Plant Cell* 11, 781–791.

Ben-Naim O, Eshed R, Parnis A, Teper-Bamnolker P, Shalit A, Coupland G, Samach A, Lifschitz E. 2006. The CCAAT binding factor can mediate interactions between CONSTANS-like proteins and DNA. *The Plant Journal* 46, 462–476.

Bhattacharjee S, Zamora A, Azhar MT, Sacco MA, Lambert LH, Moffett P. 2009. Virus resistance induced by NB–LRR proteins involves Argonaute4-dependent translational control. *The Plant Journal* 58, 940–951.

Bhattacharyya RP, Reményi A, Yeh BJ, Lim WA. 2006. Domains, motifs, and scaffolds: the role of modular interactions in the evolution and wiring of cell signaling circuits. *Annual Review of Biochemistry* 75, 655–680.

Bilgin DD, Liu Y, Schiff M, Dinesh-Kumar S. 2003. P58IPK, a plant ortholog of double-stranded RNA-dependent protein kinase PKR inhibitor, functions in viral pathogenesis. *Developmental Cell* 4, 651–661.

Bisgrove SR, Lee Y-RJ, Liu B, Peters NT, Kropf DL. 2008. The microtubule plus-end binding protein EB1 functions in root responses to touch and gravity signals in *Arabidopsis*. *The Plant Cell* 20, 396–410.

Blake-Hodek KA, Cassimeris L, Huffaker TC. 2010. Regulation of microtubule dynamics by Bim1 and Bik1, the budding yeast members of the EB1 and CLIP-170 families of plus-end tracking proteins. *Molecular Biology of the Cell* 21, 2013–2023.

Blanc S, Ammar ED, Garcia-Lampasona S, Dolja VV, Llave C, Baker J, Pirone TP. 1998. Mutations in the potyvirus helper component protein: effects on interactions with virions and aphid stylets. *Journal of General Virology* 79, 3119–3122.

Bokros CL, Hugdahl JD, Kim HH, Hanesworth VR, Van Heerden A, Browning KS, Morejohn LC. 1995. Function of the p86 subunit of eukaryotic initiation factor (iso) 4F as a microtubule-associated protein in plant cells. *Proceedings of the National Academy of Sciences of the United States of America* 92, 7120-7124.

Bowie AG, Unterholzner L. 2008. Viral evasion and subversion of pattern-recognition receptor signalling. *Nature Reviews. Immunology* 8, 911–922.

Boyko V, Ferralli J, Heinlein M. 2000. Cell-to-cell movement of TMV RNA is temperature-dependent and corresponds to the association of movement protein with microtubules. *The Plant Journal* 22, 315–325.
Branden C, Tooze J. 1999. Introduction to Protein Structure. 2nd ed. Garland Publishing Inc, New York, USA.
Brandner K, Sambade A, Boutant E, Didier P, Mély Y, Ritzenthaler C, Heinlein M. 2008. *Tobacco mosaic virus* movement protein interacts with green fluorescent protein-tagged microtubule end-binding protein 1. *Plant Physiology* 147, 611.
Brigneti G, Voinnet O, Li WX, Ji LH, Ding SW, Baulcombe DC. 1998. Viral pathogenicity determinants are suppressors of transgene silencing in *Nicotiana benthamiana*. *The EMBO Journal* 17, 6739–6746.
Buey RM, Díaz JF, Andreu JM. 2006. The nucleotide switch of tubulin and microtubule assembly: a polymerization-driven structural change. *Biochemistry* 45, 5933–5938.
Burgess J, Fleming EN. 1974. Ultrastructural observations of cell wall regeneration around isolated tobacco protoplasts. *Journal of Cell Science* 14, 439–449.
Buschmann H, Fabri CO, Hauptmann M, Hutzler P, Laux T, Lloyd CW, Schäffner AR. 2004. Helical growth of the *Arabidopsis* mutant *tortifolia1* reveals a plant-specific microtubule-associated protein. *Current Biology* 14, 1515–1521.
Cai G, Cresti M. 2010. Microtubule motors and pollen tube growth—still an open question. *Protoplasma* 247, 131–143.
Cai G, Ovidi E, Romagnoli S, Vantard M, Cresti M, Tiezzi A. 2005. Identification and characterization of plasma membrane proteins that bind to microtubules in pollen tubes and generative cells of tobacco. *Plant and Cell Physiology* 46, 563 –578.
Canto T, Palukaitis P. 1999. The hypersensitive response to *Cucumber mosaic virus* in *Chenopodium amaranticolor* requires virus movement outside the initially infected cell. *Virology* 265, 74–82.
Caplan JL, Mamillapalli P, Burch-Smith TM, Czymmek K, Dinesh-Kumar SP. 2008. Chloroplastic protein NRIP1 mediates innate immune receptor recognition of a viral effector. *Cell* 132, 449–462.
Caplan JL, Zhu X, Mamillapalli P, Marathe R, Anandalakshmi R, Dinesh-Kumar SP. 2009. Induced ER chaperones regulate a receptor-like kinase to mediate antiviral innate immune response in plants. *Cell Host & Microbe* 6, 457–469.
Carlsbecker A, Lee J-Y, Roberts CJ, Dettmer J, Lehesranta S, Zhou J, Lindgren O, Moreno-Risueno MA, Vatén A, Thitamadee S, Campilho A, Sebastian J, Bowman JL, Helariutta Y, Benfey PN. 2010. Cell signalling by microRNA165/6 directs gene dose-dependent root cell fate. *Nature* 465, 316–321.
Carrasco P, Iglesia F de la, Elena SF. 2007. Distribution of fitness and virulence effects caused by single-nucleotide substitutions in *Tobacco etch virus*. *Journal of Virology* 81, 12979–12984.
Carrington JC, Cary SM, Parks TD, Dougherty WG. 1989. A second proteinase encoded by a plant potyvirus genome. *The EMBO Journal* 8, 365–370.
Chaerle L, Lenk S, Hagenbeek D, Buschmann C, Van Der Straeten D. 2007. Multicolor fluorescence imaging for early detection of the hypersensitive reaction to *Tobacco mosaic virus*. *Journal of Plant Physiology* 164, 253–262.
Chan J, Calder G, Fox S, Lloyd C. 2007. Cortical microtubule arrays undergo rotary movements in *Arabidopsis* hypocotyl epidermal cells. *Nature Cell Biology* 9, 171–175.
Chan J, Calder GM, Doonan JH, Lloyd CW. 2003. EB1 reveals mobile microtubule nucleation sites in *Arabidopsis*. *Nature Cell Biology* 5, 967–971.
Chan J, Sambade A, Calder G, Lloyd C. 2009. *Arabidopsis* cortical microtubules are initiated along, as well as branching from existing microtubules. *The Plant Cell* 21, 2298-2306.
Cheng YQ, Liu ZM, Xu J, Zhou T, Wang M, Chen YT, Li HF, Fan ZF. 2008. HC-Pro protein of *Sugar cane mosaic virus* interacts specifically with maize ferredoxin-5 *in vitro* and *in planta*. *Journal of General Virology* 89, 2046- 2054.
Chernov K, Curmi P, Hamon L, Mechulam A, Ovchinnikov L, Pastre D. 2008. Atomic force microscopy reveals binding of mRNA to microtubules mediated by two major mRNP proteins YB-1 and PABP. *FEBS Letters* 582, 2875–2881.
Chiba S, Miyanishi M, Andika IB, Kondo H, Tamada T. 2008. Identification of amino acids of the beet necrotic yellow vein virus p25 protein required for induction of the resistance response in leaves of *Beta vulgaris* plants. *Journal of General Virology* 89, 1314–1323.
Chiera JM, Lindbo JA, Finer JJ. 2008. Quantification and extension of transient GFP expression by the co-introduction of a suppressor of silencing. *Transgenic research* 17, 1143–1154.

Cho S-Y, Cho WK, Choi H-S, Kim K-H. 2012. Cis-acting element (SL1) of *Potato virus X* controls viral movement by interacting with the NbMPB2Cb and viral proteins. *Virology* 427, 166–176.

Chowda-Reddy RV, Sun H, Hill JH, Poysa V, Wang A. 2011. Simultaneous mutations in multi-viral proteins are required for *Soybean mosaic virus* to gain virulence on soybean genotypes carrying different R genes. *PLoS One* 6, e28342.

Christensen N, Tilsner J, Bell K, Hammann P, Parton R, Lacomme C, Oparka K. 2009. The 5′ cap of *Tobacco mosaic virus* (TMV) is required for virion attachment to the actin/endoplasmic reticulum network during early infection. *Traffic* 10, 536–551.

Chuong SDX, Good AG, Taylor GJ, Freeman MC, Moorhead GBG, Muench DG. 2004. Large-scale identification of tubulin-binding proteins provides insight on subcellular trafficking, metabolic channeling, and signaling in plant cells. *Molecular & Cellular Proteomics* 3, 970.

Chuong SDX, Franceschi VR, Edwards GE. 2006. The cytoskeleton maintains organelle partitioning required for single-cell C4 photosynthesis in Chenopodiaceae species. *The Plant Cell* 18, 2207–2223.

Cole C, Barber JD, Barton GJ. 2008. The Jpred 3 secondary structure prediction server. *Nucleic Acids Research* 36, W197–W201.

Combet C, Blanchet C, Geourjon C, Deléage G. 2000. NPS@: network protein sequence analysis. *Trends in Biochemical Sciences* 25, 147–150.

Conti E, Müller CW, Stewart M. 2006. Karyopherin flexibility in nucleocytoplasmic transport. *Current Opinion in Structural Biology* 16, 237–244.

Cosson P, Sofer L, Le QH, Léger V, Schurdi-Levraud V, Whitham SA, Yamamoto ML, Gopalan S, Le Gall O, Candresse T, Carrington JC, Revers F. 2010. *RTM3*, which controls long-distance movement of potyviruses, is a member of a new plant gene family encoding a Meprin and TRAF homology domain-containing protein. *Plant Physiology* 154, 222–232.

Costet L, Fritig B, Kauffmann S. 2002. Scopoletin expression in elicitor-treated and *Tobacco mosaic virus*-infected tobacco plants. *Physiologia Plantarum* 115, 228–235.

Cotton S, Grangeon R, Thivierge K, Mathieu I, Ide C, Wei T, Wang A, Laliberté J-F. 2009. *Turnip mosaic virus* RNA replication complex vesicles are mobile, align with microfilaments, and are each derived from a single viral genome. *Journal of Virology* 83, 10460–10471.

Crowell EF, Bischoff V, Desprez T, Rolland A, Stierhof Y-D, Schumacher K, Gonneau M, Höfte H, Vernhettes S. 2009. Pausing of Golgi bodies on microtubules regulates secretion of cellulose synthase complexes in *Arabidopsis*. *The Plant Cell* 21, 1141–1154.

Culver JN, Dawson WO. 1991. Tobacco mosaic virus elicitor coat protein genes produce a hypersensitive phenotype in transgenic *Nicotiana sylvestris* plants. *Molecular Plant-Microbe Interactions* 4, 458–463.

Culver-Hanlon TL, Lex SA, Stephens AD, Quintyne NJ, King SJ. 2006. A microtubule-binding domain in dynactin increases dynein processivity by skating along microtubules. *Nature Cell Biology* 8, 264–270.

Curin M, Ojangu EL, Trutnyeva K, Ilau B, Truve E, Waigmann E. 2007. MPB2C, a microtubule-associated plant factor, is required for microtubular accumulation of *Tobacco mosaic virus* movement protein in plants. *Plant Physiology* 143, 801.

Currie JD, Stewman S, Schimizzi G, Slep KC, Ma A, Rogers SL. 2011. The microtubule lattice and plus-end association of *Drosophila* Mini spindles is spatially regulated to fine-tune microtubule dynamics. *Molecular Biology of the Cell* 22, 4343–4361.

Cyr RJ, Palevitz BA. 1995. Organization of cortical microtubules in plant cells. *Current Opinion in Cell Biology* 7, 65–71.

Dawson WO, Lehto KM. 1990. Regulation of tobamovirus gene expression. *Advances in Virus Research* 38, 307–342.

Deeks MJ, Fendrych M Smertenko A Bell KS, Oparka K Cvrckova F, Zarsky V, Hussey PJ. 2010. The plant formin AtFH4 interacts with both actin and microtubules, and contains a newly identified microtubule-binding domain. *Journal of Cell Science* 123, 1209–1215.

Delgado-Eckert E, Ojosnegros S, Beerenwinkel N. 2011. The evolution of virulence in RNA viruses under a competition–colonization trade-off. *Bulletin of Mathematical Biology* 73, 1881–1908.

De Smet I, Voss U, Jürgens G, Beeckman T. 2009. Receptor-like kinases shape the plant. *Nature Cell Biology* 11, 1166–1173.

Diaz-Pendon JA, Truniger V, Nieto C, Garcia-Mas J, Bendahmane A, Aranda MA. 2004. Advances in understanding recessive resistance to plant viruses. *Molecular Plant Pathology* 5, 223–233.

Dielen A-S, Sassaki FT, Walter J, Michon T, MéNard G, Pagny G, Krause-Sakate R, Maia IDG, Badaoui S, Le Gall O, Candresse T, German-Retana S. 2011. The 20S proteasome α5 subunit of *Arabidopsis thaliana* carries an RNase activity and interacts in planta with the lettuce mosaic potyvirus HcPro protein. *Molecular Plant Pathology* 12, 137–150.

Dinesh-Kumar SP, Tham WH, Baker BJ, 2000. Structure-function analysis of the *Tobacco mosaic virus* resistance gene *N*. *Proceedings of the National Academy of Sciences of the United States of America* 97, 14789–14794.

Dodding MP, Way M. 2011. Coupling viruses to dynein and kinesin-1. *The EMBO Journal* 30, 3527–3539.

Dolja VV, Herndon KL, Pirone TP, Carrington JC. 1993. Spontaneous mutagenesis of a plant potyvirus genome after insertion of a foreign gene. *Journal of Virology* 67, 5968–5975.

Domingo E, Holland JJ. 1997. RNA virus mutations and fitness for survival. *Annual Review of Microbiology* 51, 151–178.

Dorey S, Baillieul F, Pierrel M-A, Saindrenan P, Fritig B, Kauffmann S. 1997. Spatial and temporal induction of cell death, defense genes, and accumulation of salicylic acid in tobacco leaves reacting hypersensitively to a fungal glycoprotein elicitor. *Molecular Plant-Microbe Interactions* 10, 646–655.

Dougherty WG, Carrington JC. 1988. Expression and function of potyviral gene products *Annual Review of Phytopathology* 26, 123–143.

Drevensek S, Goussot M, Duroc Y, Christodoulidou A, Steyaert S, Schaefer E, Duvernois E, Grandjean O, Vantard M, Bouchez D, Pastuglia M. 2012. The *Arabidopsis* TRM1-TON1 interaction reveals a recruitment network common to plant cortical microtubule arrays and eukaryotic centrosomes. Plant Cell 24, 178–191.

Dryková D, Cenklová V, Sulimenko V, Volc J, Dráber P, Binarová P. 2003. Plant γ-tubulin interacts with αβ-tubulin dimers and forms membrane-associated complexes. *The Plant Cell* 15, 465 –480.

Dufresne PJ, Thivierge K, Cotton S, Beauchemin C, Ide C, Ubalijoro E, Laliberté J-F, Fortin MG. 2008. Heat shock 70 protein interaction with *Turnip mosaic virus* RNA-dependent RNA polymerase within virus-induced membrane vesicles Virology 374, 217–227.

Dunoyer P, Lecellier C-H, Parizotto EA, Himber C, Voinnet O. 2004. Probing the microRNA and small interfering RNA pathways with virus-encoded suppressors of RNA silencing. *The Plant Cell* 16, 1235–1250.

Duprat A, Caranta C, Revers F, Menand B, Browning KS, Robaglia C. 2002. The *Arabidopsis* eukaryotic initiation factor (iso) 4E is dispensable for plant growth but required for susceptibility to potyviruses. *The Plant Journal* 32, 927–934.

Ebhardt HA, Thi EP, Wang MB, Unrau PJ. 2005. Extensive 3′ modification of plant small RNAs is modulated by helper component-proteinase expression. *Proceedings of the National Academy of Sciences of the United States of America* 102, 13398–13403.

Eggenberger AL, Hajimorad MR, Hill JH. 2008. Gain of virulence on *Rsv1*-genotype soybean by an avirulent *Soybean mosaic virus* requires concurrent mutations in both P3 and HC-Pro. *Molecular Plant-Microbe Interactions* 21, 931–936.

Ehrhardt DW. 2008. Straighten up and fly right—microtubule dynamics and organization of non-centrosomal arrays in higher plants. *Current Opinion in Cell Biology* 20, 107–116.

Elena SF, Bedhomme S, Carrasco P, Cuevas JM, De la Iglesia F, Lafforgue G, Lalic J, Pròsper À, Tromas N, Zwart MP. 2011. The evolutionary genetics of emerging plant RNA viruses. *Molecular Plant-Microbe Interactions* 24, 287–293.

Endres MW, Gregory BD, Gao Z, Foreman AW, Mlotshwa S, Ge X, Pruss GJ, Ecker JR, Bowman LH, Vance V. 2010. Two plant viral suppressors of silencing require the ethylene-inducible host transcription factor RAV2 to block RNA silencing. *PLoS Pathogens* 6, e1000729.

Eskelin K, Hafrén A, Rantalainen KI, Mäkinen K. 2011. Potyviral VPg enhances viral RNA translation and inhibits reporter mRNA translation *in planta*. *Journal of Virology* 85, 9210–9221.

Eskelin K, Suntio T, Hyvärinen S, Hafrén A, Mäkinen K. 2010. *Renilla* luciferase-based quantitation of Potato virus A infection initiated with *Agrobacterium* infiltration of *N. benthamiana* leaves. *Journal of Virological Methods* 164, 101–110.

Faurez F, Baldwin T, Tribodet M, Jacquot E. 2012. Identification of new *Potato virus Y* (PVY) molecular determinants for the induction of vein necrosis in tobacco. *Molecular Plant Pathology* 13, 948–959.

Ferralli J, Ashby J, Fasler M, Boyko V, Heinlein M. 2006. Disruption of microtubule organization and centrosome function by expression of *Tobacco mosaic virus* movement protein. *Journal of Virology* 80, 5807.

Flynn DC. 2001. Adaptor proteins. *Oncogene* 20, 6270–6272.

Frey N, Klotz J, Nick P. 2010. A kinesin with calponin-homology domain is involved in premitotic nuclear migration. *Journal of Experimental Botany* 61, 3423–3437.

Friedrich L, Vernooij B, Gaffney T, Morse A, Ryals J. 1995. Characterization of tobacco plants expressing a bacterial salicylate hydroxylase gene. *Plant Molecular Biology* 29, 959–968.

Fu Y, Xu T, Zhu L, Wen M, Yang Z. 2009. A ROP GTPase signaling pathway controls cortical microtubule ordering and cell expansion in *Arabidopsis*. *Current Biology* 19, 1827–1832.

Furutani I, Watanabe Y, Prieto R, Masukawa M, Suzuki K, Naoi K, Thitamadee S, Shikanai T, Hashimoto T. 2000. The SPIRAL genes are required for directional control of cell elongation in *Arabidopsis thaliana*. *Development* 127, 4443-4453.

Gallie DR, Browning KS. 2001. eIF4G functionally differs from eIFiso4G in promoting internal initiation, cap-independent translation and translation of structured mRNAs. *The Journal of Biological Chemistry* 276, 36951–36960.

Gao L, Shen W, Yan P, Tuo D, Li X, Zhou P. 2012. NIa-pro of *Papaya ringspot virus* interacts with papaya methionine sulfoxide reductase B1. *Virology* 434, 78–87.

Gillespie T, Boevink P, Haupt S, Roberts AG, Toth R, Valentine T, Chapman S, Oparka KJ. 2002. Functional analysis of a DNA-shuffled movement protein reveals that microtubules are dispensable for the cell-to-cell movement of *Tobacco mosaic virus*. *The Plant Cell* 14, 1207 – 1222.

Gonzalez-Ibeas D, Cañizares J, Aranda MA. 2012. Microarray analysis shows that recessive resistance to *Watermelon mosaic virus* in melon is associated with the induction of defense response genes. *Molecular Plant-Microbe Interactions* 25, 107–118.

Gorbea C, Pratt G, Ustrell V, Bell R, Sahasrabudhe S, Hughes RE, Rechsteiner M. 2010. A protein interaction network for Ecm29 links the 26S proteasome to molecular motors and endosomal components. *The Journal of Biological Chemistry* 285, 31616–31633.

Grangeon R, Agbeci M, Chen J, Grondin G, Zheng H, Laliberté J-F. 2012. Impact on the endoplasmic reticulum and Golgi apparatus of *Turnip mosaic virus* infection. *Journal of Virology* 86, 9255–9265.

Grigoriev I, Gouveia SM, van der Vaart B, Demmers J, Smyth JT, Honnappa S, Splinter D, Steinmetz MO, Putney Jr JW, Hoogenraad CC, Akhmanova A. 2008. STIM1 is a MT-plus-end-tracking protein involved in remodeling of the ER. *Current Biology* 18, 177–182.

Groppo R, Brown BA, Palmenberg AC. 2011. Mutational analysis of the EMCV 2A protein identifies a nuclear localization signal and an eIF4E binding site. *Virology* 410, 257–267.

Grzela R, Strokovska L, Andrieu J-P, Dublet B, Zagorski W, Chroboczek J. 2006. Potyvirus terminal protein VPg, effector of host eukaryotic initiation factor eIF4E. *Biochimie* 88, 887–896.

Guan X, Buchholz G, Nick P. 2013. The cytoskeleton is disrupted by the bacterial effector HrpZ, but not by the bacterial PAMP flg22, in tobacco BY-2 cells. *Journal of Experimental Botany*

Guo B, Lin J, Ye K. 2011. Structure of the autocatalytic cysteine protease domain of potyvirus helper-component proteinase. *The Journal of Biological Chemistry* 286, 21937–21943.

Guo D, Merits A, Saarma M. 1999. Self-association and mapping of interaction domains of helper component-proteinase of potato A potyvirus. *Journal of General Virology* 80, 1127–1131.

Guo D, Rajamäki ML, Saarma M, Valkonen JPT. 2001. Towards a protein interaction map of potyviruses: protein interaction matrixes of two potyviruses based on the yeast two-hybrid system. *Journal of General Virology* 82, 935–939.

Guo D, Spetz C, Saarma M, Valkonen JPT. 2003. Two potato proteins, including a novel RING finger protein (HIP1), interact with the potyviral multifunctional protein HCpro. *Molecular Plant-Microbe Interactions* 16, 405–410.

Gutierrez R, Lindeboom JJ, Paredez AR, Emons A-MC, Ehrhardt DW. 2009. *Arabidopsis* cortical microtubules position cellulose synthase delivery to the plasma membrane and interact with cellulose synthase trafficking compartments. *Nature Cell Biology* 11, 797–806.

Haapalainen M, Engelhardt S, Küfner I, Li C-M, Nürnberger T, Lee J, Romantschuk M, Taira S. 2011. Functional mapping of harpin HrpZ of *Pseudomonas syringae* reveals the sites responsible for protein oligomerization, lipid interactions and plant defence induction. *Molecular Plant Pathology* 12, 151–166.

Hafrén A, Hofius D, Rönnholm G, Sonnewald U, Mäkinen K. 2010. HSP70 and its cochaperone CPIP promote Potyvirus infection in *Nicotiana benthamiana* by regulating viral coat protein functions. *The Plant Cell* 22, 523 –535.

Hajimorad MR, Eggenberger AL, Hill JH. 2008. Adaptation of *Soybean mosaic virus* avirulent chimeras containing P3 sequences from virulent strains to *Rsv1*-genotype soybeans is mediated by mutations in HC-Pro. *Molecular Plant-Microbe Interactions* 21, 937–946.

Hamada H, Takeuchi S, Kiba A, Tsuda S, Suzuki K, Hikichi Y, Okuno T. 2005. Timing and extent of hypersensitive response are critical to restrict local and systemic spread of *Pepper mild mottle virus* in pepper containing the L^3 gene. *Journal of General Plant Pathology* 71, 90–94.

Hamada T, Igarashi H, Taguchi R, Fujiwara M, Fukao Y, Shimmen T, Yokota E, Sonobe S. 2009. The putative RNA-processing protein, THO2, is a microtubule-associated protein in tobacco. *Plant and Cell Physiology* 50, 801-811.

Hamada T, Tominaga M, Fukaya T, Nakamura M, Nakano A, Watanabe Y, Hashimoto T, Baskin TI. 2012. RNA processing bodies, peroxisomes, Golgi bodies, mitochondria, and ER tubule junctions frequently pause at cortical microtubules. *Plant and Cell Physiology* 53, 699-708

Hardham AR. 2013. Microtubules and biotic interactions. *The Plant Journal* 75, 278–289.

Harries P, Ding B. 2011. Cellular factors in plant virus movement: At the leading edge of macromolecular trafficking in plants *Virology* 411, 237–243.

Harries PA, Palanichelvam K, Yu W, Schoelz JE, Nelson RS. 2009. The *Cauliflower mosaic virus* protein P6 forms motile inclusions that traffic along actin microfilaments and stabilize microtubules. *Plant Physiology* 149, 1005–1016.

Harries PA, Schoelz JE, Nelson RS. 2010. Intracellular transport of viruses and their components: utilizing the cytoskeleton and membrane highways. *Molecular Plant-Microbe Interactions* 23, 1381–1393.

Hashimoto T. 2002. Molecular genetic analysis of left–right handedness in plants *PhilosophicalTransactions of the Royal Society of London. Series B, Biological Sciences* 357, 799-808.

Hawley RS, Gilliland WD. 2006. Sometimes the result is not the answer: the truths and the lies that come from using the complementation test. *Genetics* 174, 5–15.

Hébrard E, Poulicard N, Gérard C, Traoré O, Wu H-C, Albar L, Fargette D, Bessin Y, Vignols F. 2010. Direct interaction between the *Rice yellow mottle virus* (RYMV) VPg and the central domain of the rice eIF(iso)4G1 factor correlates with rice susceptibility and RYMV virulence. *Molecular Plant-Microbe Interactions* 23, 1506–1513.

Heinlein M, Epel BL, Padgett HS, Beachy RN. 1995. Interaction of tobamovirus movement proteins with the plant cytoskeleton. *Science* 270, 1983–1985.

Heinrich M, Hettenhausen C, Lange T, Wünsche H, Fang J, Baldwin IT, Wu J. 2012. High levels of jasmonic acid antagonize the biosynthesis of gibberellins and inhibit the growth of *Nicotiana attenuata* stems. *The Plant Journal* 73, 591-606.

Higaki T, Kurusu T, Hasezawa S, Kuchitsu K. 2011. Dynamic intracellular reorganization of cytoskeletons and the vacuole in defense responses and hypersensitive cell death in plants. *Journal of Plant Research* 124, 315–324.

Hoefle C, Huesmann C, Schultheiss H, Börnke F, Hensel G, Kumlehn J, Hückelhoven R. 2011. A barley ROP GTPase activating protein associates with microtubules and regulates entry of the barley powdery mildew fungus into leaf epidermal cells. *The Plant Cell* 23, 2422-2439.

Hofius D, Maier AT, Dietrich C, Jungkunz I, Börnke F, Maiss E, Sonnewald U. 2007. Capsid protein-mediated recruitment of host DnaJ-Like proteins is required for *Potato virus Y* infection in tobacco plants. *Journal of Virology* 81, 11870–11880.

Huang T-S, Wei T, Laliberté J-F, Wang A. 2010. A host RNA helicase-like protein, AtRH8, interacts with the potyviral genome-linked protein, VPg, associates with the virus accumulation complex, and is essential for infection. *Plant Physiology* 152, 255-266.

Huang Z, Yeakley JM, Garcia EW, Holdridge JD, Fan J-B, Whitham SA. 2005. Salicylic acid-dependent expression of host genes in compatible *Arabidopsis*-virus interactions. *Plant Physiology* 137, 1147–1159.

Hunt RS. 1994. Comment on the letter by Andrivon - Re: Pathogenicity and virulence. *Phytopathology* 84, 874–875.

Hämäläinen JH, Kekarainen T, Gebhardt C, Watanabe KN, Valkonen JPT. 2000. Recessive and dominant genes interfere with the vascular transport of *Potato virus A* in diploid potatoes. *Molecular Plant-Microbe Interactions* 13, 402–412.

Höög JL, Schwartz C, Noon AT, O'Toole ET, Mastronarde DN, McIntosh JR, Antony C. 2007. Organization of interphase microtubules in fission yeast analyzed by electron tomography. *Developmental Cell* 12, 349–361.

ICTV. 2012. Virus Taxonomy, Ninth Report of the International Committee on Taxonomy of Viruses. King AMQ, Adams MJ, Carstens EB Lefkowitz EJ (Eds.). Elsevier Academic, London, UK.

Ishibashi K, Masuda K, Naito S, Meshi T, Ishikawa M. 2007. An inhibitor of viral RNA replication is encoded by a plant resistance gene. *Proceedings of the National Academy of Sciences of the United States of America* 104, 13833–13838.

Ishida T, Kaneko Y, Iwano M, Hashimoto T. 2007. Helical microtubule arrays in a collection of twisting tubulin mutants of *Arabidopsis thaliana*. *Proceedings of the National Academy of Sciences of the United States of America* 104, 8544–8549.

Jamous RM, Boonrod K, Fuellgrabe MW, Ali-Shtayeh MS, Krczal G, Wassenegger M. 2011. The helper component-proteinase of the *Zucchini yellow mosaic virus* inhibits the Hua Enhancer 1 methyltransferase activity *in vitro*. *Journal of General Virology* 92, 2222 –2226.

Janzac B, Montarry J, Palloix A, Navaud O, Moury B. 2010. A point mutation in the polymerase of *Potato virus Y* confers virulence toward the *Pvr4* resistance of pepper and a high competitiveness cost in susceptible cultivar. *Molecular Plant-Microbe Interactions* 23, 823–830.

Jenner CE, Wang X, Ponz F, Walsh JA. 2002. A fitness cost for *Turnip mosaic virus* to overcome host resistance. *Virus Research* 86, 1–6.

Jin Y, Ma D, Dong J, Jin J, Li D, Deng C, Wang T. 2007a. HC-Pro protein of *Potato virus Y* can interact with three *Arabidopsis* 20S proteasome subunits *in planta*. *Journal of Virology* 81, 12881–12888.

Jin Y, Ma D, Dong J, Li D, Deng C, Jin J, Wang T. 2007b. The HC-pro protein of *Potato virus Y* interacts with NtMinD of tobacco. *Molecular Plant-Microbe Interactions* 20, 1505–1511.

Johansen LK, Carrington JC. 2001. Silencing on the spot Induction and suppression of RNA silencing in the *Agrobacterium*-mediated transient expression system. *Plant Physiology* 126, 930–938.

Jones JDG, Dangl JL. 2006. The plant immune system. *Nature* 444, 323–329.

Kaneda M, Rensing K, Samuels L. 2010. Secondary cell wall deposition in developing secondary xylem of poplar. *Journal of Integrative Plant Biology* 52, 234–243.

Kang B-C, Yeam I, Frantz JD, Murphy JF, Jahn MM. 2005. The *pvr1* locus in *Capsicum* encodes a translation initiation factor eIF4E that interacts with *Tobacco etch virus* VPg. *The Plant Journal* 42, 392–405.

Kang H-G, Kuhl JC, Kachroo P, Klessig DF. 2008. CRT1, an Arabidopsis ATPase that interacts with diverse resistance proteins and modulates disease resistance to *Turnip crinkle virus*. *Cell Host & Microbe* 3, 48–57.

Kang H-G, Oh C-S, Sato M, Katagiri F, Glazebrook J, Takahashi H, Kachroo P, Martin GB, Klessig DF. 2010. Endosome-associated CRT1 functions early in resistance gene–mediated defense signaling in *Arabidopsis* and tobacco. *The Plant Cell* 22, 918–936.

Kang H-G, Woo Choi H, von Einem S, Manosalva P, Ehlers K, Liu P-P, Buxa SV, Moreau M, Mang H-G, Kachroo P, Kogel K-H, Klessig DF. 2012. CRT1 is a nuclear-translocated MORC endonuclease that participates in multiple levels of plant immunity. *Nature Communications* 3, 1297.

Kang SH, Lim WS, Kim KH. 2004. A protein interaction map of *Soybean mosaic virus* strain G7H based on the yeast two-hybrid system. *Molecules and Cells* 18, 122–126.

Kappel C, Zachariae U, Dölker N, Grubmüller H. 2010. An unusual hydrophobic core confers extreme flexibility to HEAT repeat proteins Biophysical Journal 99, 1596–1603.

Kasschau KD, Carrington JC. 1998. A counterdefensive strategy of plant viruses: suppression of posttranscriptional gene silencing. *Cell* 95, 461–470.

Kasschau KD, Carrington JC. 2001. Long-distance movement and replication maintenance functions correlate with silencing suppression activity of potyviral HC-Pro. *Virology* 285, 71–81.

Kasschau KD, Cronin S, Carrington JC. 1997. Genome amplification and long-distance movement functions associated with the central domain of tobacco etch potyvirus helper component-proteinase *Virology* 228, 251–262.

Kawamura E, Himmelspach R, Rashbrooke MC, Whittington AT, Gale KR, Collings DA, Wasteneys GO. 2006. MICROTUBULE ORGANIZATION 1 regulates structure and function

of microtubule arrays during mitosis and cytokinesis in the *Arabidopsis* root. *Plant Physiology* 140, 102–114.
Kawamura E, Wasteneys GO. 2008. MOR1, the *Arabidopsis thaliana* homologue of *Xenopus* MAP215, promotes rapid growth and shrinkage, and suppresses the pausing of microtubules *in vivo*. *Journal of Cell Science* 121, 4114 –4123.
Keech O, Pesquet E, Gutierrez L, Ahad A, Bellini C, Smith SM, Gardeström P. 2010. Leaf senescence is accompanied by an early disruption of the microtubule network in *Arabidopsis*. *Plant Physiology* 154, 1710–1720.
Kekarainen T, Merits A, Oruetxebarria I, Rajamäki M-L, Valkonen JPT. 1999. Comparison of the complete sequences of five different isolates of *Potato virus A* (PVA), genus *Potyvirus*. *Archives of Virology* 144, 2355–2366.
Kerppola TK. 2006. Design and implementation of bimolecular fluorescence complementation (BiFC) assays for the visualization of protein interactions in living cells. *Nature Protocols* 1, 1278–1286.
Kieft JS. 2008. Viral IRES RNA structures and ribosome interactions. *Trends in Biochemical Sciences* 33, 274–283.
Kim J-G, Li X, Roden JA, Taylor KW, Aakre CD, Su B, Lalonde S, Kirik A, Chen Y, Baranage G, McLane H, Martin GB, Mudgett MB. 2009. *Xanthomonas* T3S effector XopN suppresses PAMP-triggered immunity and interacts with a tomato atypical receptor-like kinase and TFT1. *The Plant Cell* 21, 1305–1323.
Kippert F, Gerloff DL. 2009. Highly sensitive detection of individual HEAT and ARM repeats with HHpred and COACH. *PLoS One* 4, e7148.
Kirik V, Herrmann U, Parupalli C, Sedbrook JC, Ehrhardt DW, Hülskamp M. 2007. CLASP localizes in two discrete patterns on cortical microtubules and is required for cell morphogenesis and cell division in *Arabidopsis*. *Journal of Cell Science* 120, 4416–4425.
Knutson BA. 2010. Insights into the domain and repeat architecture of target of rapamycin. *Journal of Structural Biology* 170, 354–363.
Komis G, Illés P, Beck M, Šamaj J. 2011. Microtubules and mitogen-activated protein kinase signalling. *Current Opinion in Plant Biology* 14, 650–657.
Krtková, J, Zimmermann A, Schwarzerová, K, Nick P. 2012. Hsp90 binds microtubules and is involved in the reorganization of the microtubular network in angiosperms. *Journal of Plant Physiology* 169, 1329–1339.
Kumar P, Wittmann T. 2012. +TIPs: SxIPping along microtubule ends. *Trends in Cell Biology* 22, 418–428.
Lacroix C, Glais L, Kerlan C, Verrier J-L, Jacquot E. 2010. Biological characterization of French *Potato virus Y* (PVY) isolates collected from PVY-susceptible or -resistant tobacco plants possessing the recessive resistance gene *va*. *Plant Pathology* 59, 1133–1143.
Lacroix C, Glais L, Verrier J-L, Charlier C, Lorencetti C, Jacquot E. 2011. Impact of tobacco recessive resistance gene *va* on biological properties of Brazilian *Potato virus Y* (PVY) isolates. *Plant Pathology* 60, 1048–1054.
Le Gall O, Christian P, Fauquet CM, King AMQ, Knowles NJ, Nakashima N, Stanway G, Gorbalenya AE. 2008. *Picornavirales*, a proposed order of positive-sense single-stranded RNA viruses with a pseudo-T = 3 virion architecture. *Archives of Virology* 153, 715–727.
Lechner B, Rashbrooke MC, Collings DA, Eng RC, Kawamura E, Whittington AT, Wasteneys GO. 2012. The N-terminal TOG domain of Arabidopsis MOR1 modulates affinity for microtubule polymers. *Journal of Cell Science* 125, 4812–4821.
Ledbetter MC, Porter KR. 1963. A "microtubule" in plant cell fine structure. *The Journal of Cell Biology* 19, 239–250.
Lee AH-Y, Hurley B, Felsensteiner C, Yea C, Ckurshumova W, Bartetzko V, Wang PW, Quach V, Lewis JD, Liu YC, Börnke F, Angers S, Wilde A, Guttman DS, Desveaux D. 2012. A bacterial acetyltransferase destroys plant microtubule networks and blocks secretion. *PLoS Pathogens* 8, e1002523.
Lehto K, Tikkanen M, Hiriart J-B, Paakkarinen V, Aro E-M. 2003. Depletion of the photosystem II core complex in mature tobacco leaves infected by the *Flavum* strain of *Tobacco mosaic virus*. *Molecular Plant-Microbe Interactions* 16, 1135–1144.
Lellis AD, Allen ML, Aertker AW, Tran JK, Hillis DM, Harbin CR, Caldwell C, Gallie DR, Browning KS. 2010. Deletion of the eIFiso4G subunit of the *Arabidopsis* eIFiso4F translation initiation complex impairs health and viability. *Plant Molecular Biology* 74, 249–263.

Lellis AD, Kasschau KD, Whitham SA, Carrington JC. 2002. Loss-of-susceptibility mutants of *Arabidopsis thaliana* reveal an essential role for eIF(iso)4E during potyvirus infection. *Current Biology* 12, 1046–1051.

Léonard S, Plante D, Wittmann S, Daigneault N, Fortin MG, Laliberté, J-F. 2000. Complex formation between *Potyvirus* VPg and translation eukaryotic initiation factor 4E correlates with virus infectivity. *Journal of Virology* 74, 7730–7737.

Léonard S, Viel C, Beauchemin C, Daigneault N, Fortin MG, Laliberté, J-F. 2004. Interaction of VPg-Pro of *Turnip mosaic virus* with the translation initiation factor 4E and the poly(A)-binding protein *in planta*. *Journal of General Virology* 85, 1055–1063.

Leopold PL, Pfister KK. 2006. Viral strategies for intracellular trafficking: motors and microtubules. *Traffic* 7, 516–523.

Lewis JD, Wu R, Guttman DS, Desveaux D. 2010. Allele- specific virulence attenuation of the *Pseudomonas syringae* HopZ1a type III effector via the *Arabidopsis* ZAR1 resistance protein. *PLoS Genetics* 6, e1000894.

Li H, Mao T, Zhang Z, Yuan M. 2007. The AtMAP65-1 cross-bridge between microtubules is formed by one dimer. *Plant and Cell Physiology* 48, 866.

Li Y, Shen Y, Cai C, Zhong C, Zhu L, Yuan M, Ren H. 2010. The type II *Arabidopsis* formin14 interacts with microtubules and microfilaments to regulate cell division. *The Plant Cell* 22, 2710.

Lin L, Shi Y, Luo Z, Lu Y, Zheng H, Yan F, Chen Jiong, Chen Jianping, Adams MJ, Wu Y. 2009. Protein–protein interactions in two potyviruses using the yeast two-hybrid system. *Virus Research* 142, 36–40.

Liu Y, Schiff M, Dinesh-Kumar SP. 2004. Involvement of MEK1 MAPKK, NTF6 MAPK, WRKY/MYB transcription factors, *COI1* and *CTR1* in *N*-mediated resistance to tobacco mosaic virus. *The Plant Journal* 38, 800–809.

Liu Y, Schiff M, Marathe R, Dinesh-Kumar SP. 2002. Tobacco *Rar1*, *EDS1* and *NPR1/NIM1* like genes are required for *N*-mediated resistance to tobacco mosaic virus. *The Plant Journal* 30, 415–429.

Love AJ, Yun BW, Laval V, Loake GJ, Milner JJ. 2005. *Cauliflower mosaic virus*, a compatible pathogen of *Arabidopsis*, engages three distinct defense-signaling pathways and activates rapid systemic generation of reactive oxygen species. *Plant Physiology* 139, 935–948.

Lózsa R, Csorba T, Lakatos L, Burgyán J. 2008. Inhibition of 3′ modification of small RNAs in virus-infected plants require spatial and temporal co-expression of small RNAs and viral silencing-suppressor proteins. *Nucleic Acids Research* 36, 4099–4107.

Lupas A, Van Dyke M, Stock J. 1991. Predicting coiled coils from protein sequences. *Science* 252, 1162–1164.

Mallory AC, Reinhart BJ, Bartel D, Vance VB, Bowman LH. 2002. A viral suppressor of RNA silencing differentially regulates the accumulation of short interfering RNAs and micro-RNAs in tobacco. *Proceedings of the National Academy of Sciences of the United States of America* 99, 15228-15233.

Mandadi KK, Scholthof K-BG. 2013. Plant immune responses against viruses: how does a virus cause disease? *The Plant Cell* doi: 101105/tpc113111658.

Marcotrigiano J, Gingras A-C, Sonenberg N, Burley SK. 1999. Cap-dependent translation initiation in eukaryotes is regulated by a molecular mimic of eIF4G. *Molecular Cell* 3, 707–716.

Marcotrigiano J, Lomakin IB, Sonenberg N, Pestova TV, Hellen CUT, Burley SK. 2001. A conserved HEAT domain within eIF4G directs assembly of the translation initiation machinery. *Molecular Cell* 7, 193–203.

Martinière A, Bak A, Macia J-L, Lautredou N, Gargani D, Doumayrou J, Garzo E, Moreno A, Fereres A, Blanc S, Drucker M. 2013. A virus responds instantly to the presence of the vector on the host and forms transmission morphs. *Elife* 2, e00183.

Martinière A, Gargani D, Uzest M, Lautredou N, Blanc S, Drucker M. 2009. A role for plant microtubules in the formation of transmission-specific inclusion bodies of *Cauliflower mosaic virus*. *The Plant Journal* 58, 135–146.

Masuta C, Nishimura M, Morishita H, Hataya T. 1999. A single amino acid change in viral genome-associated protein of *Potato virus Y* correlates with resistance breaking in 'Virgin A Mutant' tobacco. *Phytopathology* 89, 118–123.

Mathur J, Mathur N, Kernebeck B, Srinivas BP, Hülskamp M. 2003. A novel localization pattern for an EB1-like protein links microtubule dynamics to endomembrane organization. *Current Biology* 13, 1991–1997 .

Matros A, Amme S, Kettig B, Buck-Sorlin GH, Sonnewald U, Mock HP. 2006. Growth at elevated CO_2 concentrations leads to modified profiles of secondary metabolites in tobacco cv. SamsunNN and to increased resistance against infection with *Potato virus Y*. *Plant, Cell & Environment* 29, 126–137.

Merits A, Rajamäki M-L, Lindholm P, Runeberg-Roos P, Kekarainen T, Puustinen P, Mäkeläinen K, Valkonen JPT, Saarma M. 2002. Proteolytic processing of potyviral proteins and polyprotein processing intermediates in insect and plant cells. *Journal of General Virology* 83, 1211–1221.

Miller S, Krijnse-Locker J. 2008. Modification of intracellular membrane structures for virus replication. *Nature Reviews. Microbiology* 6, 363–374.

Min BE, Martin K, Wang R, Tafelmeyer P, Bridges M, Goodin M. 2010. A host-factor interaction and localization map for a plant-adapted *Rhabdovirus* implicates cytoplasm-tethered transcription activators in cell-to-cell movement. *Molecular Plant-Microbe Interactions* 23, 1420–1432.

Mishina TE, Zeier J. 2007. Pathogen-associated molecular pattern recognition rather than development of tissue necrosis contributes to bacterial induction of systemic acquired resistance in *Arabidopsis* .*The Plant Journal* 50, 500–513.

Mitchison T, Kirschner M. 1984. Dynamic instability of microtubule growth. *Nature* 312, 237–242.

Miyoshi H, Okade H, Muto S, Suehiro N, Nakashima H, Tomoo K, Natsuaki T. 2008. *Turnip mosaic virus* VPg interacts with *Arabidopsis thaliana* eIF(iso)4E and inhibits *in vitro* translation. *Biochimie* 90, 1427–1434.

Mock HP, Heller W, Molina A, Neubohn B, Sandermann H Jr, Grimm B. 1999. Expression of *uroporphyrinogen decarboxylase* or *coproporphyrinogen oxidase* antisense RNA in tobacco induces pathogen defense responses conferring increased resistance to *Tobacco mosaic virus*. *The Journal of Biological Chemistry* 274, 4231.

Motose H, Hamada T, Yoshimoto K, Murata T, Hasebe M, Watanabe Y, Hashimoto T, Sakai T, Takahashi T. 2011. NIMA-related kinases 6, 4, and 5 interact with each other to regulate microtubule organization during epidermal cell expansion in *Arabidopsis thaliana*. *The Plant Journal* 67, 993–1005.

Moury B, Caromel B, Johansen E, Simon V, Chauvin L, Jacquot E, Kerlan C, Lefebvre V. 2011. The helper component-proteinase cistron of *Potato virus Y* induces hypersensitivity and resistance in potato genotypes carrying dominant resistance genes on chromosome IV. *Molecular Plant-Microbe Interactions* 24, 787–797.

Moutevelis E, Woolfson DN. 2009. A periodic table of coiled-coil protein structures. *Journal of Molecular Biology* 385, 726–732.

Murata T, Sonobe S, Baskin TI, Hyodo S, Hasezawa S, Nagata T, Horio T, Hasebe M. 2005. Microtubule-dependent microtubule nucleation based on recruitment of γ-tubulin in higher plants. *Nature Cell Biology* 7, 961–968.

Nakajima K, Furutani I, Tachimoto H, Matsubara H, Hashimoto T. 2004. SPIRAL1 encodes a plant-specific microtubule-localized protein required for directional control of rapidly expanding *Arabidopsis* cells. *The Plant Cell* 16, 1178–1190.

Nakamura M, Hashimoto T. 2009. A mutation in the *Arabidopsis* gamma-tubulin-containing complex causes helical growth and abnormal microtubule branching. *Journal of Cell Science* 122, 2208–2217.

Nakaseko Y, Nabeshima K, Kinoshita K, Yanagida M. 1996. Dissection of fission yeast microtubule associating protein p93Dis1: regions implicated in regulated localization and microtubule interaction. *Genes to Cells: Devoted to Molecular & Cellular Mechanisms* 1, 633–644.

Naoi K, Hashimoto T. 2004. A semidominant mutation in an *Arabidopsis* mitogen-activated protein kinase phosphatase-like gene compromises cortical microtubule organization. *The Plant Cell* 16, 1841–1853.

Nicaise V, German-Retana S, Sanjuán R, Dubrana M-P, Mazier M, Maisonneuve B, Candresse T, Caranta C, Le Gall O. 2003. The eukaryotic translation initiation factor 4E controls lettuce susceptibility to the *Potyvirus Lettuce mosaic virus*. *Plant Physiology* 132, 1272–1282.

Nick P. 2013. Microtubules, signalling and abiotic stress. *The Plant Journal* 75, 309–323.

Nicolas O, Dunnington SW, Gotow LF, Pirone TP, Hellmann GM. 1997. Variations in the VPg protein allow a potyvirus to overcome *va* gene resistance in tobacco. *Virology* 237, 452–459.

Nie X. 2006. Salicylic acid suppresses *Potato virus Y* isolate N:O-induced symptoms in tobacco plants. *Phytopathology* 96, 255–263.

Niehl A, Amari K, Gereige D, Brandner K, Mély Y, Heinlein M. 2012. Control of *Tobacco mosaic virus* movement protein fate by CELL-DIVISION-CYCLE protein 48. *Plant Physiology* 160, 2093–2108.

Niehl A, Peña EJ, Amari K, Heinlein M. 2013. Microtubules in viral replication and transport. *The Plant Journal* 75, 290–308.

Oruetxebarria I, Kekarainen T, Spetz C, Valkonen JPT. 2000. Molecular characterization of *Potato virus V* genomes from Europe indicates limited spatiotemporal strain differentiation. *Phytopathology* 90, 437–444.

Ouko MO, Sambade A, Brandner K, Niehl A, Peña E, Ahad A, Heinlein M, Nick P. 2010. Tobacco mutants with reduced microtubule dynamics are less susceptible to TMV. *The Plant Journal* 62, 829–839.

Paalme V, Gammelgård E, Järvekülg L, Valkonen JPT. 2004. *In vitro* recombinants of two nearly identical potyviral isolates express novel virulence and symptom phenotypes in plants. *Journal of General Virology* 85, 739–747.

Pacheco R, García-Marcos A, Manzano A, de Lacoba MG, Camañes G, García-Agustín P, Díaz-Ruíz JR, Tenllado F. 2012. Comparative analysis of transcriptomic and hormonal responses to compatible and incompatible plant-virus interactions that lead to cell death. *Molecular Plant-Microbe Interactions* 25, 709–723.

Padmanabhan MS, Goregaoker SP, Golem S, Shiferaw H, Culver JN. 2005. Interaction of the *Tobacco mosaic virus* replicase protein with the Aux/IAA protein PAP1/IAA26 is associated with disease development. *Journal of Virology* 79, 2549–2558.

Palloix A, Ayme V, Moury B. 2009. Durability of plant major resistance genes to pathogens depends on the genetic background, experimental evidence and consequences for breeding strategies. *New Phytologist* 183, 190–199.

Pastuglia M, Bouchez D. 2007. Molecular encounters at microtubule ends in the plant cell cortex. *Current Opinion in Plant Biology* 10, 557–563.

Patel S, Dinesh-Kumar SP. 2008. *Arabidopsis* ATG6 is required to limit the pathogen-associated cell death response Autophagy 4, 20–27.

Peltier C, Schmidlin L, Klein E, Taconnat L, Prinsen E, Erhardt M, Heintz D, Weyens G, Lefebvre M, Renou J-P, Gilmer D. 2011. Expression of the *Beet necrotic yellow vein virus* p25 protein induces hormonal changes and a root branching phenotype in *Arabidopsis thaliana*. *Transgenic Research* 20, 443–466.

Peng YH, Kadoury D, Gal-On A, Huet H, Wang Y, Raccah B. 1998. Mutations in the HC-Pro gene of *Zucchini yellow mosaic Potyvirus*: effects on aphid transmission and binding to purified virions. *Journal of General Virology* 79, 897–904.

Pineda M, Gáspár L, Morales F, Szigeti Z, Barón M. 2008. Multicolor fluorescence imaging of leaves–a useful tool for visualizing systemic viral infections in plants *Photochemistry and Photobiology* 84, 1048–1060.

Plisson C, Drucker M, Blanc S, German-Retana S, Le Gall O, Thomas D, Bron P. 2003. Structural characterization of HC-Pro, a plant virus multifunctional protein. *The Journal of Biological Chemistry* 278, 23753–23761.

Pontier D, Tronchet M, Rogowsky P, Lam E, Roby D. 1998. Activation of *hsr203*, a plant gene expressed during incompatible plant-pathogen interactions, is correlated with programmed cell death. *Molecular Plant-Microbe Interactions* 11, 544–554.

Pruss G, Ge X, Shi XM, Carrington JC, Vance VB. 1997. Plant viral synergism: the potyviral genome encodes a broad-range pathogenicity enhancer that transactivates replication of heterologous viruses. *The Plant Cell* 9, 859–868.

Pruss GJ, Lawrence CB, Bass T, Li QQ, Bowman LH, Vance V. 2004. The potyviral suppressor of RNA silencing confers enhanced resistance to multiple pathogens *Virology* 320, 107–120.

Purcifull DE, Hiebert E. 1992. Serological relationships involving potyviral nonstructural proteins. *Archives of Virology* Supplement 5, 97–122.

Qiao F, Chang X-L, Nick P. 2010. The cytoskeleton enhances gene expression in the response to the harpin elicitor in grapevine. *Journal of Experimental Botany* 61, 4021–4031.

Quenouille J, Montarry J, Palloix A, Moury B. 2013. Farther, slower, stronger: how the plant genetic background protects a major resistance gene from breakdown. *Molecular Plant Pathology* 14, 109–118.

Radtke K, Döhner K, Sodeik B. 2006. Viral interactions with the cytoskeleton: a hitchhiker's guide to the cell. *Cellular Microbiology* 8, 387–400.

Rajamäki M, Merits A, Rabenstein F, Andrejeva J, Paulin L, Kekarainen T, Kreuze JF, Forster RLS, Valkonen JPT. 1998. Biological, serological, and molecular differences among isolates of potato a potyvirus. *Phytopathology* 88, 311–321.

Rajamäki M-L, Mäki-Valkama T, Mäkinen K, Valkonen JPT. 2004. Infection with potyviruses. In: Talbot NJ (ed), Plant-Pathogen Interactions. Blackwell Publishing, Sheffield, UK. pp 68–91.

Rajamäki M-L, Valkonen JPT. 1999. The 6K2 protein and the VPg of *Potato virus A* are determinants of systemic infection in *Nicandra physaloides*. *Molecular Plant-Microbe Interactions* 12, 1074–1081.

Rajamäki M-L, Valkonen JPT. 2002. Viral genome-linked protein (VPg) controls accumulation and phloem-loading of a potyvirus in inoculated potato leaves. *Molecular Plant-Microbe Interactions* 15, 138–149.

Rajamäki M-L, Valkonen JPT. 2009. Control of nuclear and nucleolar localization of nuclear inclusion protein A of *Picorna*-like *Potato virus A* in *Nicotiana* species. *The Plant Cell* 21, 2485 –2502.

Rantalainen KI, Christensen PA, Hafrén A, Otzen DE, Kalkkinen N, Mäkinen K. 2009. Interaction of a potyviral VPg with anionic phospholipid vesicles. *Virology* 395, 114–120.

Ratcliff F, Martin-Hernandez AM, Baulcombe DC. 2001. Technical advance. *Tobacco rattle virus* as a vector for analysis of gene function by silencing. *The Plant Journal* 25, 237–245.

Redondo E, Krause-Sakate R, Yang SJ, Lot H, Le Gall O, Candresse T. 2001. *Lettuce mosaic virus* pathogenicity determinants in susceptible and tolerant lettuce cultivars map to different regions of the viral genome. *Molecular Plant-Microbe Interactions* 14, 804–810.

Reichel C, Beachy RN. 1998. Tobacco mosaic virus infection induces severe morphological changes of the endoplasmic reticulum. *Proceedings of the National Academy of Sciences of the United States of America* 95, 11169–11174.

Reinbothe S, Mollenhauer B, Reinbothe C. 1994. JIPs and RIPs: the regulation of plant gene expression by jasmonates in response to environmental cues and pathogens. *The Plant Cell* 6, 1197–1209.

Rhoads RE. 2009. eIF4E: new family members, new binding partners, new roles. *The Journal of Biological Chemistry* 284, 16711–16715.

Richter JD, Sonenberg N. 2005. Regulation of cap-dependent translation by eIF4E inhibitory proteins. *Nature* 433, 477–480.

Robatzek S. 2007. Vesicle trafficking in plant immune responses Cellular Microbiology 9, 1–8.

Rodríguez M, Muñoz N, Lenardon S, Lascano R. 2012. The chlorotic symptom induced by *Sunflower chlorotic mottle virus* is associated with changes in redox-related gene expression and metabolites. *Plant Science* 196, 107–116.

Rojas MR, Zerbini FM, Allison RF, Gilbertson RL, Lucas WJ. 1997. Capsid protein and helper component-proteinase function as potyvirus cell-to-cell movement proteins *Virology* 237, 283–295.

Roudet-Tavert G, Michon T, Walter J, Delaunay T, Redondo E, Le Gall O. 2007. Central domain of a potyvirus VPg is involved in the interaction with the host translation initiation factor eIF4E and the viral protein HcPro. *Journal of General Virology* 88, 1029.

Roulin A, Auer PL, Libault M, Schlueter J, Farmer A, May G, Stacey G, Doerge RW, Jackson SA. 2013. The fate of duplicated genes in a polyploid plant genome. *The Plant Journal* 73, 143–153.

Roy A, Kucukural A, Zhang Y. 2010. I-TASSER: a unified platform for automated protein structure and function prediction Nat Protoc 5, 725–738.

Ruffel S, Dussault M-H, Palloix A, Moury B, Bendahmane A, Robaglia C, Caranta C. 2002. A natural recessive resistance gene against *Potato virus Y* in pepper corresponds to the eukaryotic initiation factor 4E (eIF4E). *The Plant Journal* 32, 1067–1075.

Ruggenthaler P, Fichtenbauer D, Krasensky J, Jonak C, Waigmann E. 2009. Microtubule-associated protein AtMPB2C plays a role in organization of cortical microtubules, stomata patterning, and tobamovirus infectivity. *Plant Physiology* 149, 1354–1365.

Ruiz-Ferrer V, Boskovic J, Alfonso C, Rivas G, Llorca O, Lopez-Abella D, Lopez-Moya JJ. 2005. Structural analysis of tobacco etch potyvirus HC-Pro oligomers involved in aphid transmission. *Journal of Virology* 79, 3758–3765.

Sahana N, Kaur H, Basavaraj Tena F, Jain RK, Palukaitis P, Canto T, Praveen S. 2012. Inhibition of the host proteasome facilitates *Papaya ringspot virus* accumulation and proteosomal catalytic activity is modulated by viral factor HcPro. *PLoS One* 7, e52546.

Sainsbury F, Collings DA, Mackun K, Gardiner J, Harper JDI, Marc J. 2008. Developmental reorientation of transverse cortical microtubules to longitudinal directions: a role for

actomyosin-based streaming and partial microtubule-membrane detachment. *The Plant Journal* 56, 116–131.

Salonen A, Ahola T, Kääriäinen L. 2005. Viral RNA replication in association with cellular membranes. *Current Topics in Microbiology and Immunology* 285, 139–173.

Sambade A, Brandner K, Hofmann C, Seemanpillai M, Mutterer J, Heinlein M. 2008. Transport of TMV movement protein particles associated with the targeting of RNA to plasmodesmata. *Traffic* 9, 2073–2088.

Sampathkumar A, Lindeboom JJ, Debolt S, Gutierrez R, Ehrhardt DW, Ketelaar T, Persson S. 2011. Live cell imaging reveals structural associations between the actin and microtubule cytoskeleton in *Arabidopsis*. *The Plant Cell* 23, 2302 –2313.

Schaad MC, Anderberg RJ, Carrington JC. 2000. Strain-specific interaction of the *Tobacco etch virus* NIa protein with the translation initiation factor eIF4E in the yeast two-hybrid system. *Virology* 273, 300–306.

Schaad MC, Jensen PE, Carrington JC. 1997. Formation of plant RNA virus replication complexes on membranes: role of an endoplasmic reticulum-targeted viral protein. *The EMBO Journal* 16, 4049–4059.

Schmelzer E. 2002. Cell polarization, a crucial process in fungal defence. *Trends in Plant Science* 7, 411–415.

Schuster M, Kilaru S, Fink G, Collemare J, Roger Y, Steinberg G. 2011. Kinesin-3 and dynein cooperate in long-range retrograde endosome motility along a nonuniform microtubule array. *Molecular Biology of the Cell* 22, 3645 –3657.

Sedbrook JC, Ehrhardt DW, Fisher SE, Scheible W-R, Somerville CR. 2004. The *Arabidopsis SKU6/SPIRAL1* gene encodes a plus end–localized microtubule-interacting protein involved in directional cell expansion. *The Plant Cell* 16, 1506–1520.

Shand K, Theodoropoulos C, Stenzel D, Dale JL, Harrison MD. 2009. Expression of *Potato virus Y* cytoplasmic inclusion protein in tobacco results in disorganization of parenchyma cells, distortion of epidermal cells, and induces mitochondrial and chloroplast abnormalities, formation of membrane whorls and atypical lipid accumulation. Micron 40, 730–736.

Shemyakina EA, Erokhina TN, Gorshkova EN, Schiemann J, Solovyev AG, Morozov SY. 2011. Formation of protein complexes containing plant virus movement protein TGBp3 is necessary for its intracellular trafficking. *Biochimie* 93, 742–748.

Shi F-M, Yao L-L, Pei B-L, Zhou Q, Li X-L, Li Y, Li Y-Z. 2009. Cortical microtubule as a sensor and target of nitric oxide signal during the defence responses to *Verticillium dahliae* toxins in *Arabidopsis*. *Plant, Cell & Environment* 32, 428–438.

Shiboleth YM, Haronsky E, Leibman D, Arazi T, Wassenegger M, Whitham SA, Gaba V, Gal-On A. 2007. The conserved FRNK box in HC-Pro, a plant viral suppressor of gene silencing, is required for small RNA binding and mediates symptom development. *Journal of Virology* 81, 13135–13148.

Shoji T, Narita NN, Hayashi K, Asada J, Hamada T, Sonobe S, Nakajima K, Hashimoto T. 2004. Plant-specific microtubule-associated protein SPIRAL2 is required for anisotropic growth in *Arabidopsis*. *Plant Physiology* 136, 3933–3944.

Shukla DD, Ward CW, Brunt AA. 1994. The *Potyviridae*. CAB International, Wallingford, UK.

Siddiqui SA, Sarmiento C, Truve E, Lehto H, Lehto K. 2008. Phenotypes and functional effects caused by various viral RNA silencing suppressors in transgenic *Nicotiana benthamiana* and *N. tabacum*. *Molecular Plant-Microbe Interactions* 21, 178–187.

Singh RP, Valkonen JPT, Gray SM, Boonham N, Jones RAC, Kerlan C, Schubert J. 2008. Discussion paper: The naming of *Potato virus Y* strains infecting potato. *Archives of Virology* 153, 1–13.

Slep KC, Vale RD. 2007. Structural basis of microtubule plus end tracking by XMAP215, CLIP-170, and EB1. *Molecular Cell* 27, 976–991.

Smertenko A, Franklin-Tong VE. 2011. Organisation and regulation of the cytoskeleton in plant programmed cell death. *Cell Death and Differentiation* 18, 1263–1270.

Soitamo A, Jada B, Lehto K. 2011. HC-Pro silencing suppressor significantly alters the gene expression profile in tobacco leaves and flowers. *BMC Plant Biology* 11, 68.

Spetz C, Taboada AM, Darwich S, Ramsell J, Salazar LF, Valkonen JPT. 2003. Molecular resolution of a complex of potyviruses infecting solanaceous crops at the centre of origin in Peru. *Journal of General Virology* 84, 2565–2578.

Steinmetz MO, Akhmanova A. 2008. Capturing protein tails by CAP-Gly domains. *Trends in Biochemical Sciences* 33, 535–545.

Stoppin-Mellet V, Gaillard J, Timmers T, Neumann E, Conway J, Vantard M. 2007. *Arabidopsis* katanin binds microtubules using a multimeric microtubule-binding domain. *Plant Physiology and Biochemistry* 45, 867–877.

Söding J, Biegert A, Lupas AN. 2005. The HHpred interactive server for protein homology detection and structure prediction. *Nucleic Acids Research* 33, W244–W248.

Takagi S, Takamatsu H, Sakurai-Ozato N. 2009. Chloroplast anchoring: its implications for the regulation of intracellular chloroplast distribution. *Journal of Experimental Botany* 60, 3301–3310.

Tarutani Y, Morimoto T, Sasaki A, Yasuda M, Nakashita H, Yoshida S, Yamaguchi I, Suzuki Y. 2004a. Molecular characterization of two highly homologous receptor-like kinase genes, *RLK902* and *RKL1*, in *Arabidopsis thaliana*. *Bioscience, Biotechnology, and Biochemistry* 68, 1935–1941.

Tarutani Y, Sasaki A, Yasuda M, Nakashita H, Yoshida S, Yamaguchi I, Suzuki Y. 2004b. Identification of three clones which commonly interact with the kinase domains of highly homologous two receptor-like kinases, RLK902 and RKL1. *Bioscience, Biotechnology, and Biochemistry* 68, 2581–2587.

Tatineni S, McMechan AJ, Hein GL, French R. 2011. Efficient and stable expression of GFP through Wheat streak mosaic virus-based vectors in cereal hosts using a range of cleavage sites: Formation of dense fluorescent aggregates for sensitive virus tracking. *Virology* 410, 268–281.

Tavert-Roudet G, Abdul-Razzak A, Doublet B, Walter J, Delaunay T, German-Retana S, Michon T, Le Gall O, Candresse T. 2012. The C terminus of lettuce mosaic potyvirus cylindrical inclusion helicase interacts with the viral VPg and with lettuce translation eukaryotic initiation factor 4E. *Journal of General Virology* 93, 184–193.

Taylor KW, Kim J-G, Su XB, Aakre CD, Roden JA, Adams CM, Mudgett MB. 2012. Tomato TFT1 is required for PAMP-triggered immunity and mutations that prevent T3S effector XopN from binding to TFT1 attenuate *Xanthomonas* virulence. *PLoS Pathogens* 8, e1002768.

Thiel H, Hleibieh K, Gilmer D, Varrelmann M. 2012. The P25 pathogenicity factor of *Beet necrotic yellow vein virus* targets the sugar beet 26S proteasome involved in the induction of a hypersensitive resistance response via interaction with an F-box protein. *Molecular Plant-Microbe Interactions* 25, 1058–1072.

Thiel H, Varrelmann M. 2009. Identification of *Beet necrotic yellow vein virus* P25 pathogenicity factor-interacting sugar beet proteins that represent putative virus targets or components of plant resistance. *Molecular Plant-Microbe Interactions* 22, 999–1010.

Thomas CL, Jones L, Baulcombe DC, Maule AJ. 2001. Size constraints for targeting post-transcriptional gene silencing and for RNA-directed methylation in *Nicotiana benthamiana* using a potato virus X vector. *The Plant Journal* 25, 417–425.

Thomma BPHJ, Nurnberger T, Joosten MHAJ. 2011. Of PAMPs and effectors: the blurred PTI-ETI dichotomy. *The Plant Cell* 23, 4–15.

Thornbury DW, Hellmann GM, Rhoads RE, Pirone TP. 1985. Purification and characterization of potyvirus helper component. Virology 144, 260–267.

Tian Y-P, Valkonen JPT. 2013. Genetic determinants of *Potato virus Y* required to overcome or trigger hypersensitive resistance to PVY strain group O controlled by the gene *Ny* in potato. *Molecular Plant-Microbe Interactions* 26, 297–305.

Torres-Barceló, C, Daròs J-A, Elena SF. 2010a. Compensatory molecular evolution of HC-Pro, an RNA-silencing suppressor from a plant RNA virus. *Molecular Biology and Evolution* 27, 543–551.

Torres-Barceló, C, Daròs J-A, Elena SF. 2010b. HC-Pro hypo- and hypersuppressor mutants: differences in viral siRNA accumulation *in vivo* and siRNA binding activity *in vitro*. *Archives of Virology* 155, 251–254.

Torres-Barceló, C, Martín S, Daròs J-A, Elena SF. 2008. From hypo- to hypersuppression: effect of amino acid substitutions on the RNA-silencing suppressor activity of the tobacco etch potyvirus HC-Pro. *Genetics* 180, 1039–1049.

Tribodet M, Glais L, Kerlan C, Jacquot E. 2005. Characterization of *Potato virus Y* (PVY) molecular determinants involved in the vein necrosis symptom induced by PVY^N isolates in infected *Nicotiana tabacum* cv Xanthi. *Journal of General Virology* 86, 2101–2105.

Tör M, Lotze MT, Holton N. 2009. Receptor-mediated signalling in plants: molecular patterns and programmes. *Journal of Experimental Botany* 60, 3645–3654.

Ueki S, Citovsky V. 2011. To gate, or not to gate: regulatory mechanisms for intercellular protein transport and virus movement in plants. *Molecular Plant* 4, 782–793.

Urcuqui-Inchima S, Maia IG, Arruda P, Haenni A-L, Bernardi F. 2000. Deletion mapping of the potyviral helper component-proteinase reveals two regions involved in RNA binding. *Virology* 268, 104–111.

Urcuqui-Inchima S, Walter J, Drugeon G, German-Retana S, Haenni A-L, Candresse T, Bernardi F, Le Gall O. 1999. Potyvirus helper component-proteinase self-interaction in the yeast two-hybrid system and delineation of the interaction domain involved. *Virology* 258, 95–99.

Valkonen JPT, Puurand Ü, Slack S, Mäkinen K, Saarma M. 1995. Three strain groups of potato A potyvirus based on hypersensitive responses in potato, serological properties, and coat protein sequences. *Plant disease* 79, 748–753.

Valkonen JPT, Rajamäki M-L, Kekarainen T. 2002. Mapping of viral genomic regions important in cross-protection between strains of a potyvirus. *Molecular Plant-Microbe Interactions* 15, 683–692.

Valkonen JPT. 1997. Novel resistances to four potyviruses in tuber-bearing potato species, and temperature-sensitive expression of hypersensitive resistance to *Potato virus Y*. *Annals of Applied Biology* 130, 91–104.

Van der Biezen EA, Jones JDG. 1998. Plant disease-resistance proteins and the gene-for-gene concept. *Trends in Biochemical Sciences* 23, 454–456.

Wang A, Krishnaswamy S. 2012. Eukaryotic translation initiation factor 4E-mediated recessive resistance to plant viruses and its utility in crop improvement. *Molecular Plant Pathology* 13, 795–803.

Wang S, Kurepa J, Hashimoto T, Smalle JA. 2011. Salt stress–induced disassembly of Arabidopsis cortical microtubule arrays involves 26S proteasome–dependent degradation of SPIRAL1. *The Plant Cell* 23, 3412–3427.

Ward BM. 2011. The taking of the cytoskeleton one two three: how viruses utilize the cytoskeleton during egress. *Virology* 411, 244–250.

Wargo AR, Kurath G. 2012. Viral fitness: definitions, measurement, and current insights. *Current Opinion in Virology* 2, 538–545.

Varrelmann M, Maiss E, Pilot R, Palkovics L. 2007. Use of pentapeptide-insertion scanning mutagenesis for functional mapping of the *Plum pox virus* helper component proteinase suppressor of gene silencing. *Journal of General Virology* 88, 1005–1015.

Vatén A, Dettmer J, Wu S, Stierhof Y-D, Miyashima S, Yadav SR, Roberts CJ, Campilho A, Bulone V, Lichtenberger R, Lehesranta S, Mähönen AP, Kim J-Y, Jokitalo E, Sauer N, Scheres B, Nakajima K, Carlsbecker A, Gallagher KL, Helariutta Y. 2011. Callose biosynthesis regulates symplastic trafficking during root development. *Developmental Cell* 21, 1144–1155.

Watson P, Forster R, Palmer KJ, Pepperkok R, Stephens DJ. 2005. Coupling of ER exit to microtubules through direct interaction of COPII with dynactin. *Nature Cell Biology* 7, 48–55.

Vaughan KT. 2005. Microtubule plus ends, motors, and traffic of Golgi membranes. *Biochimica et Biophysica Acta* 1744, 316–324.

Wei T, Huang T-S, McNeil J, Laliberté, J-F, Hong J, Nelson RS, Wang A. 2010. Sequential recruitment of the endoplasmic reticulum and chloroplasts for plant potyvirus replication. *Journal of Virology* 84, 799–809.

Wei T, Wang A. 2008. Biogenesis of cytoplasmic membranous vesicles for plant potyvirus replication occurs at endoplasmic reticulum exit sites in a COPI- and COPII-dependent manner. *Journal of Virology* 82, 12252–12264.

Wen R-H, Khatabi B, Ashfield T, Maroof MS, Hajimorad MR. 2013. The HC-Pro and P3 cistrons of an avirulent *Soybean mosaic virus* are recognized by different resistance genes at the complex *Rsv1* locus. *Molecular Plant-Microbe Interactions* 26, 203–215.

Verchot J. 2011. Wrapping membranes around plant virus infection. *Current Opinion in Virology* 1, 388–395.

Whitham S, Dinesh-Kumar SP, Choi D, Hehl R, Corr C, Baker B. 1994. The product of the *Tobacco mosaic virus* resistance gene *N*: Similarity to toll and the interleukin-1 receptor. *Cell* 78, 1101–1115.

Whitham SA, Anderberg RJ, Chisholm ST, Carrington JC. 2000. *Arabidopsis RTM2* gene is necessary for specific restriction of *Tobacco etch virus* and encodes an unusual small heat shock–like protein. *The Plant Cell* 12, 569–582.

Whitham SA, Quan S, Chang H-S, Cooper B, Estes B, Zhu T, Wang X, Hou Y-M. 2003. Diverse RNA viruses elicit the expression of common sets of genes in susceptible *Arabidopsis thaliana* plants. *The Plant Journal* 33, 271–283.

Vick JK, Nebenführ A. 2012. Putting on the breaks: regulating organelle movements in plant cells. *Journal of Integrative Plant Biology* 54, 868–874.

Widlund PO, Stear JH, Pozniakovsky A, Zanic M, Reber S, Brouhard GJ, Hyman AA, Howard J. 2011. XMAP215 polymerase activity is built by combining multiple tubulin-binding TOG domains and a basic lattice-binding region. *Proceedings of the National Academy of Sciences of the United States of America* 108, 2741 –2746.

Wileman T. 2006. Aggresomes and autophagy generate sites for virus replication. *Science* 312, 875–878.

Winter N, Kollwig G, Zhang S, Kragler F. 2007. MPB2C, a microtubule-associated protein, regulates non-cell-autonomy of the homeodomain protein KNOTTED1. *The Plant Cell* 19, 3001–3018.

Wittmann S, Chatel H, Fortin MG, Laliberté, J-F. 1997. Interaction of the viral protein genome linked of turnip mosaic potyvirus with the translational eukaryotic initiation factor (iso) 4E of *Arabidopsis thaliana* using the yeast two-hybrid system. *Virology* 234, 84–92.

Vogel F, Hofius D, Sonnewald U. 2007. Intracellular trafficking of *Potato leafroll virus* movement protein in transgenic *Arabidopsis*. *Traffic* 8, 1205–1214.

Voigt B, Timmers ACJ, Šamaj J, Hlavacka A, Ueda T, Preuss M, Nielsen E, Mathur J, Emans N, Stenmark H, Nakano A, Baluška F, Menzel D. 2005. Actin-based motility of endosomes is linked to the polar tip growth of root hairs. *European Journal of Cell Biology* 84, 609–621.

Wright KM, Cowan GH, Lukhovitskaya NI, Tilsner J, Roberts AG, Savenkov EI, Torrance L. 2010. The N-terminal domain of PMTV TGB1 movement protein is required for nucleolar localization, microtubule association, and long-distance movement. *Molecular Plant-Microbe Interactions* 23, 1486–1497.

Vuorinen AL, Gammelgård E, Auvinen P, Somervuo P, Dere S, Valkonen JPT. 2010. Factors underpinning the responsiveness and higher levels of virus resistance realised in potato genotypes carrying virus-specific *R* genes. *Annals of Applied Biology* 157, 229–241.

Vuorinen AL, Kelloniemi J, Valkonen JPT. 2011. Why do viruses need phloem for systemic invasion of plants? *Plant Science* 181, 355–363.

Yalovsky S, Bloch D, Sorek N, Kost B. 2008. Regulation of membrane trafficking, cytoskeleton dynamics, and cell polarity by ROP/RAC GTPases. *Plant Physiology* 147, 1527–1543.

Yambao M, Leonora M, Masuta C, Nakahara K, Uyeda I. 2003. The central and C-terminal domains of VPg of *Clover yellow vein virus* are important for VPg-HCPro and VPg-VPg interactions. *Journal of General Virology* 84, 2861.

Yambao MLM, Yagihashi H, Sekiguchi H, Sekiguchi T, Sasaki T, Sato M, Atsumi G, Tacahashi Y, Nakahara KS, Uyeda I. 2007. Point mutations in helper component protease of *Clover yellow vein virus* are associated with the attenuation of RNA-silencing suppression activity and symptom expression in broad bean. *Archives of Virology* 153, 105–115.

Yang C, Guo R, Jie F, Nettleton D, Peng J, Carr T, Yeakley JM, Fan J-B, Whitham SA. 2007. Spatial analysis of *Arabidopsis thaliana* gene expression in response to *Turnip mosaic virus* infection. *Molecular Plant-Microbe Interactions* 20, 358–370.

Yang C, Zhang C, Dittman JD, Whitham SA. 2009. Differential requirement of ribosomal protein S6 by plant RNA viruses with different translation initiation strategies *Virology* 390, 163–173.

Yang D-L, Yao J, Mei C-S, Tong X-H, Zeng L-J, Li Q, Xiao L-T, Sun T-P, Li J, Deng X-W, Lee CM, Thomashow MF, Yang Y, He Z, He SY. 2012. Plant hormone jasmonate prioritizes defense over growth by interfering with gibberellin signaling cascade. *Proceedings of the National Academy of Sciences of the United States of America* 109, E1192–E1200.

Yao L, Zhou Q, Pei B, Li Y. 2011. Hydrogen peroxide modulates the dynamic microtubule cytoskeleton during the defence responses to *Verticillium dahliae* toxins in *Arabidopsis*. *Plant, Cell & Environment* 34, 1586–1598.

Yao M, Wakamatsu Y, Itoh TJ, Shoji T, Hashimoto T. 2008. *Arabidopsis* SPIRAL2 promotes uninterrupted microtubule growth by suppressing the pause state of microtubule dynamics. *Journal of Cell Science* 121, 2372–2381.

Yoshii M, Nishikiori M, Tomita K, Yoshioka N, Kozuka R, Naito S, Ishikawa M. 2004. The *Arabidopsis cucumovirus multiplication 1* and *2* loci encode translation initiation factors 4E and 4G. *Journal of Virology* 78, 6102-6111.

Yu B, Chapman EJ, Yang Z, Carrington JC, Chen X. 2006. Transgenically expressed viral RNA silencing suppressors interfere with microRNA methylation in *Arabidopsis*. *FEBS Letters* 580, 3117–3120.

Zamyatnin AA Jr, Solovyev AG, Bozhkov PV, Valkonen JPT, Morozov SY, Savenkov EI. 2006. Assessment of the integral membrane protein topology in living cells. *The Plant Journal* 46, 145–154.

Zhang C, Kotchoni SO, Samuels AL, Szymanski DB. 2010. SPIKE1 signals originate from and assemble specialized domains of the endoplasmic reticulum. *Current Biology* 20, 2144–2149.

Zhang Y, Skolnick J. 2004. Scoring function for automated assessment of protein structure template quality. *Proteins: Structure, Function, and Bioinformatics* 57, 702–710.

Zhang Y, Skolnick J. 2005. TM-align: a protein structure alignment algorithm based on the TM-score. *Nucleic Acids Research* 33, 2302–2309.

Zheng H, Yan F, Lu Y, Sun L, Lin L, Cai L, Hou M, Chen J. 2011. Mapping the self-interacting domains of TuMV HC-Pro and the subcellular localization of the protein. *Virus Genes* 42, 110–116.

Zhu S, Gao F, Cao X, Chen M, Ye G, Wei C, Li Y. 2005. The *Rice dwarf virus* P2 protein interacts with *ent*-kaurene oxidases in vivo, leading to reduced biosynthesis of gibberellins and rice dwarf symptoms. *Plant Physiology* 139, 1935 –1945.

Zilian E, Maiss E. 2011. Detection of plum pox potyviral protein-protein interactions *in planta* using an optimized mRFP-based bimolecular fluorescence complementation system. *Journal of General Virology* 92, 2711–2723.

Zizlsperger N, Keating AE. 2010. Specific coiled-coil interactions contribute to a global model of the structure of the spindle pole body. *Journal of Structural Biology* 170, 246–256.

Zvereva AS, Pooggin MM. 2012. Silencing and innate immunity in plant defense against viral and non-viral pathogens. *Viruses* 4, 2578–2597.